



APPEAL BRIEF  
EXAMINING GROUP 1632  
Patent Application  
Docket No. GJE-21D2  
Serial No. 09/760,274


IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner : Michael C. Wilson  
Art Unit : 1632  
Applicants : John Sinden, Jeffrey A. Gray, Helen Hodges, Timothy Kershaw,  
Fiza Rashid-Doubell  
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For : Neural Transplantation Using Pluripotent Neuroepithelial Cells

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**APPEAL BRIEF**

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transplantation improves brain function of the mammal, as recited in  
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1. Appellants' specification, which broadly teaches that pluripotent, nestin-positive neuroepithelial cells may be obtained from various areas of the brain and intracerebrally transplanted to treat a disorder associated with damage to, or loss of, brain cells, and exemplifies using mouse hippocampal pluripotent, nestin-positive neuroepithelial cells to treat a cognitive deficit associated with damage to, or loss of brain cells in the hippocampus, enables claims that are not limited to transplantation of mouse hippocampal cells to treat a cognitive deficit associated with damage to, or loss of, hippocampal cells. ....18

2. Appellants' specification, which broadly teaches that conditionally immortal pluripotent, nestin-positive neuroepithelial cells may be transplanted to any portion of the brain, and exemplifies transplanting conditionally immortal pluripotent, nestin-positive neuroepithelial cells to the hippocampus, enables claims that are not limited to transplantation to the hippocampus. . ....20

3. Appellants' specification, which broadly teaches that conditionally immortal pluripotent, nestin-positive neuroepithelial cells may be intracerebrally transplanted into a mammal, such as a human, to treat a disorder associated with damage to, or loss of, brain cells, and exemplifies transplanting conditionally immortal pluripotent, nestin-positive neuroepithelial cells to a rat model, enables claims that are not limited to transplantation to a rat. ....23

4. Appellants' specification, which broadly teaches that pluripotent, nestin-positive neuroepithelial cells may be genetically modified to be conditionally immortal, such that the cells are immortal prior to transplantation and differentiate after transplantation, and exemplifies transduction with a temperature-sensitive simian virus 40 large T antigen under the control of an interferon-inducible H-2K<sup>b</sup> promoter, enables claims that are not limited to the temperature-sensitive simian virus 40 large T antigen under the control of the interferon-inducible H-2K<sup>b</sup> promoter. ....26

## APPENDICES

Appendix A (Claims Appendix)

Appendix B (Evidence Appendix)



I. REAL PARTY IN INTEREST

This application is owned by ReNeuron Limited.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences. Accordingly, no Related Proceedings appendix is part of the Brief.

III. STATUS OF CLAIMS

Claims 57, 58, 60-62, 64, and 76-86 are pending in the application and are under final rejection. Claims 1-48, 59, and 68-75 were cancelled in the Amendment dated February 20, 2004. Claims 49-56 were cancelled in the Amendment dated September 30, 2002. Claims 63 and 65-67 were cancelled in the Amendment dated March 20, 2003. The rejections of claims 57, 58, 60-62, 64, and 76-86 are appealed herein.

IV. STATUS OF AMENDMENTS

A Response to the final Office Action dated May 25, 2004, was filed on October 21, 2004. The Response of October 21, 2004 was entered. Claims 57, 58, 60-62, 64, and 76-86 are currently pending and attached hereto as Appendix A.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claims 57, 81, and 85 are independent. Appellants' invention pertains to novel methods for treating a disorder associated with damage to, or loss of, brain cells in a mammal, by intracerebrally transplanting pluripotent, nestin-positive, neuroepithelial cells into the brain of the mammal, thereby improving the mammal's brain function. Intracerebral transplantation is discussed at page 14, lines 16-32, of the specification of application Serial No. 09/760,274, hereinafter referred to as the '274 application. The cells to be transplanted in each of claims 57, 81, and 85 are pluripotent and conditionally immortal. As "pluripotent" cells, they have not yet completed differentiation into a specific, terminally differentiated cell type, instead having the potential to further differentiate into different types or different phenotypes of cell (see, for example, page 1, lines 22-30, of the '274 application). The pluripotent cells are nestin-positive, as taught in Example 4, at page 20, lines 4-37, and page 21, lines 1-10, of the '274 application.

Further, these cells have been genetically modified to be conditionally immortal, such that they are immortal prior to transplantation and differentiate after transplantation (see, for example page 5, lines 32-36; page 6, lines 1-36; page 7, lines 1-7, and page 12, lines 23-31, of the '274 application). One method of rendering the cells conditionally immortal is by transduction of an oncogene, such as the temperature-sensitive simian virus 40 large T antigen, described at page 9, lines 1-13, of the '274 application, and recited in claims 81 and 85. The oncogene may be under the control of a promoter such as the interferon-inducible H-2K<sup>b</sup> promoter, recited in claim 85. The pluripotent cells may be human cells, as taught at page 7, lines 8-10, and page 13, lines 12-16, of the '274 application, and recited in claims 81 and 85. When transplanted into the brain of a mammal suffering from a disorder associated with damage to, or loss of, brain cells, the pluripotent neuroepithelial cells respond to signals from the affected brain by taking up a phenotype that is able to replace or compensate for the functional deficit caused by the damage or loss of brain cells (see page 5, lines 10-24; page 10, lines 34-36; and page 11, lines 109, of the '274 application).

Transplantation of conspecific fetal neural tissue into a damaged brain has been studied previously in animal experiments and consequent repair has been observed at the neuroanatomical, physiological and behavioral levels (see, for example, page 4, lines 28-32, of the '274 application). Widespread use of this work within the context of Parkinson's disease has been frustrated by the need for tissue derived from conspecific fetal brain, *i.e.*, the fetal tissue required must be specific to the type of damage intended to be repaired and it must be harvested at a precise, time-limited stage during brain development that varies with brain region and cell type. Hence, the requirement for specific matching of cell types leads to both practical and ethical problems (see, for example, page 5, lines 1-9, of the '274 application). Appellants discovered that when conditionally immortal, pluripotent, nestin-positive neuroepithelial cells are implanted into a damaged brain, the cells differentiate into the appropriate cell type required to repair the brain, and the differentiated cells are able to form the appropriate neural connections required to improve function. Thus, it is not required that the pluripotent cells be obtained from the same region of the brain as the damaged region. However, this may also be done. For example, claim 85 recites that the deficit is caused by damage to the hippocampus and the pluripotent cells are hippocampal cells. The phenotype of the differentiated cells may be the same phenotype as the damaged or lost cells, or may be a different phenotype, or a number of

phenotypes. In any case, the cells take up a phenotype that is capable of functionally integrating and compensating for the damaged or lost cells (see, for example, page 5, lines 10-22, of the '274 application). Furthermore, once they are transplanted, pluripotent neuroepithelial cells have the ability to migrate extensively, seeking out damaged tissue and functionally integrating. This facilitates repair in situations where the damage is widespread or where the locus of the damage is not entirely known (see, for example, page 7, lines 21-36; page 8, lines 1-10; and Example 9 of the '274 application).

Because of the cells' plasticity and migration capability, one clonal pluripotent cell line can repair damage in a number of different areas of the brain, and if more than one particular neural cell type is required to repair damage in a given area, then a single pluripotent cell line will be capable of differentiating into the different types of neural cells required to achieve repair. Thus, the present invention provides both a strategy and a material basis for transplant therapies with which to target a wide range of behavioral and psychological deficits caused by an equally wide range of forms of damage to the brain. For example, the methods of the invention may be used to treat a cognitive deficit, as recited in claim 85 (see, for example, page 9, lines 29-35; page 10, lines 1-9; and Examples 5-9 at pages 22-29 of the '274 application).

## VI. GROUNDS OF REJECTION

A. Claims 57, 58, 60-62, 64, and 76-86 stand rejected under 35 U.S.C. § 112, first paragraph, as lacking sufficient written description.

B. Claims 57, 58, 81, 82, 85, and 86 stand rejected under 35 U.S.C. § 112, first paragraph, as new matter.

C. Claims 57, 58, 60-62, and 76-86 stand rejected under 35 U.S.C. § 112, first paragraph, as non-enabled.

## VII. ARGUMENT

**A. The specification provides sufficient written description of that which is essential to obtain human pluripotent, nestin-positive neuroepithelial cells capable of improving brain function resulting from damage to, or loss of, brain cells.**

Claims 57, 58, 60-62, 64, and 76-86 stand rejected under 35 U.S.C. §112, first paragraph, as lacking sufficient written description. As an initial matter, in the final Office Action dated May 25, 2004, the Examiner indicated that the rejection of claims 57, 58, and 60-62, for lack of written description, regarding transplanting “human” pluripotent, nestin-positive neuroepithelial cells has been withdrawn because the term “human” has been deleted (page 6, lines 17-20). Appellants note that claims 57, 58, and 60-62 have not been amended subsequent to the final Office Action; nonetheless, the Advisory Action dated December 21, 2004 included claims 57, 58, and 60-62 in the instant rejection under 35 U.S.C. §112, first paragraph, as lacking sufficient written description. Appellants are understandably perplexed by the apparent inconsistency in the stated disposition of the claims under this rejection.

In the final Office Action dated May 25, 2004 and the subsequent Advisory Action dated December 21, 2004, the Examiner indicates that the ‘274 application does not provide an adequate written description of a method of using human, nestin-positive, neuroepithelial cells for treating a cognitive deficit in humans because the specification does not adequately describe the human cells capable of treating a cognitive deficit.

Thus, claiming a method of treating cognitive deficit using pluripotent, nestin-positive neuroepithelial cells in humans without defining the properties required to obtain human cells capable of treating a cognitive deficit or how to use mouse cells to treat humans is not in compliance with the written description requirement (emphasis added; Office Action dated May 25, 2004; page 8, lines 17-21).

The nestin-positive, musashi-positive, human, pluripotent, neural precursor cells described in the Declaration by Dr. Sinden as having the desired function in vivo have a narrower scope than nestin-positive, human, pluripotent, neural precursor cells described in the specification as originally filed. As such, applicants did not adequately describe that which was essential to obtain human neural precursor cells having the desired function, i.e., nestin-positive neural precursor cells expressing musashi. The experimental results in the Declaration by Dr. Sinden remain unpersuasive because the cells used in the experiment were of a narrower scope than those described in the specification as originally filed

and because musashi expression may be essential to obtain nestin-positive neural precursor cells with the desired function in humans (emphasis in original; Advisory Action dated December 21, 2004; page 3, lines 5-15).

The Office Action states that the '274 application teaches using mouse cells to restore cognitive function and suggests the use of human cells isolated at about eight weeks gestation. The Office Action cites the Gray *et al.* publication (*Philosophical Transactions of the Royal Soc. London*, 1999, 354(1388):1407-1421) as showing that fully differentiated hippocampal cells not yet having axons are essential to the invention, that the isolation of suitable cells must be taken at fifteen weeks gestation, and that this is essential to obtain the required amount of differentiation. However, the passage in the Gray *et al.* publication relied on by the Examiner concerns an overview of conventional transplantation methods using fully differentiated cells, not the pluripotent nestin-positive cells used in the method of the invention. When transplanting differentiated cells, it is clearly important that the correct phenotype of cell be selected for transplantation. Therefore, Appellants respectfully submit that the Examiner's interpretation and extrapolation of the teaching of Gray *et al.* to the pluripotent cells used in the present invention is incorrect.

In contrast to differentiated cells, which the prior art teaches must be conspecific, the cells of the present invention are pluripotent and have the ability to differentiate into different phenotypes, depending on external factors. Upon transplantation, the pluripotent cells are stimulated to differentiate into the desired phenotype (see page 5, lines 10-24, of the '274 application). Therefore, controlling differentiation is not an issue for the conditionally immortal pluripotent neuroepithelial cells used in the methods of the subject invention.

At page 10, the Office Action dated May 25, 2004 states that the applicants do not provide an adequate written description for the human equivalent of the mouse, nestin-positive cells capable of treating cognitive function. However, the subject specification teaches that the pluripotent cells used in the claimed method should be isolated early enough in the developmental pathway that they retain the ability to differentiate into the desired brain cell phenotypes (page 13, lines 7-11, of the '274 application). It is well known by those skilled in the art that the plasticity (*e.g.*, pluripotency) of embryonic cells is generally inversely related to the age of embryonic development. Therefore, if nestin-positive pluripotent cells are obtainable from a human at 12 weeks gestation (as proven in the Sinden Declaration), it is at least as likely,

if not more likely, that the cells would be obtainable from a human at an earlier stage of development, for example, at 8 weeks, as suggested in the '274 application.

As indicated at page 13, lines 5-7 of the '274 application, the region of the brain from which neuroepithelial cells are obtained and the precise time (stage and development) they are obtained may vary. Appellants point to the Declaration under 37 C.F.R. §1.132 by Dr. Sinden, dated September 27, 2002, including Exhibits A-D, of record, which accompanied the Amendment submitted on September 30, 2002. Exhibit D describes an experiment in which human pluripotent neuroepithelial cells were isolated from the fetal cortex, conditionally modified using techniques described in the '274 application (page 6, lines 11-31, and page 12, lines 10-23), and implanted into the brains of rats having unilateral basal forebrain excitotoxic lesions, a model recognized in the art as one which mimics cell loss that occurs in Alzheimer's disease and other neurodegenerative diseases. Restoration of function was assessed using a water maze test, wherein poor performance across several parameters reflects spatial long-term and short-term learning and memory impairments. The human cell line was assessed in comparison with the murine MHP36 cell line (a cell line exemplified in the '274 application) as a positive control, and with sham-grafted lesioned and non-lesioned controls. Non-lesioned controls received vehicle at the same sites. Rats grafted with the murine MHP36 cell line performed significantly better than lesioned animals. However, rats receiving cells of the human cell line showed as rapid spatial learning as non-lesioned controls, and were superior both to the lesion-only group and the murine-grafted group. The Sinden Declaration shows that pluripotent nestin-positive neuroepithelial cells can be obtained from human fetal cortex at 12 weeks gestation and restore function (see paragraph 9 and Exhibit D of the Sinden Declaration). Thus, page 13, lines 5-16, of the '274 application highlights 8 weeks as an example of when such pluripotent cells can be isolated. The human cells can also be isolated at 12 weeks (as demonstrated by Exhibit D) and even later, depending on the brain region from which cells are obtained. As will be appreciated by one of ordinary skill in the art, the hippocampus and cortex are properly identifiable as anatomical structures at approximately 10-12 weeks, which is why the human cells were isolated at the 12-week gestation period. The human cells described in Exhibit D were taken from the cortex because the cells are readily obtainable in large numbers from this brain region. The success of the experiment described in Exhibit D using cortex cells supports the teachings of the specification that pluripotent neuroepithelial cells other than

hippocampal cells are capable of improving a brain disorder, such as cognitive deficit. Once obtained, cells can then be screened for pluripotency *in vitro*, to verify their ability to differentiate upon transplantation, as taught at page 13, lines 17-28, and Example 4, at pages 20-21 of the '274 application. The Examiner has provided no reasons to doubt that the pluripotent cells used in the claimed methods can be obtained from more than one region of the brain or at more than one gestational stage.

The May 25, 2004 Office Action cites Renfranz (*Cell*, 1991, 66:713-729) for teaching that nestin-positive pluripotent cells differentiate into different neural cell lineages. The Renfranz publication teaches the establishment of a differentiated cell line derived from embryonic precursor cells. Because the cells are pluripotent, they have the ability to differentiate into different types of neural cells, depending on environmental factors. This is consistent with the properties of the pluripotent neuroepithelial cells as taught and used in the method of the invention. Upon transplantation, the pluripotent neuroepithelial cells are stimulated to differentiate into different neural phenotypes that are capable of functionally integrating and compensating for the damaged or lost cells. As taught at page 5, lines 10-31, of the '274 application, this means that with one clonal pluripotent cell line it is possible to repair damage in a number of different areas of the brain, and that if more than one particular neural cell type is required to repair damage in a given area, then a single pluripotent cell line will be capable of differentiating into the different types of neural cells required to achieve repair. This is a major advantage of the method of the invention.

The Office Action also cites the Villa *et al.* publication (*Exp. Neurol.*, 2000, 161:67-84) as showing that properties identifying human neural stem cells are not well understood. Appellants respectfully submit that the Villa *et al.* publication supports the written description of the claimed subject matter in that it shows that one of ordinary skill in the art can obtain cells having the properties that the specification teaches are desirable. The Villa *et al.* publication is concerned with defining the optimal conditions for preparing suitable pluripotent cells and makes various statements that genetically-modified cells provide the most convenient method. The cells used in the Villa *et al.* publication are taken at approximately ten weeks gestation. Furthermore, the Villa *et al.* publication makes it clear that suitable cells can be defined in terms of nestin expression and pluripotency. These are properties of the cells that are taught in the specification and recited in the claims of the subject application. Although there is a statement in

the Villa *et al.* publication that properties identifying a human neural stem cell are not well understood, this does not mean that a human neuroepithelial pluripotent cell cannot be identified. Pages 13 and 20-21 of the subject '274 application teach how to do so. Clearly, Villa *et al.* and others in the art have done so. Although the Villa *et al.* publication was not publicly available at the time of filing, because they used the same techniques as taught by Appellants, it demonstrates that the subject specification contained all the information necessary for one of ordinary skill in the art to carry out the invention.

At page 10 of the Office Action dated May 25, 2004, the Examiner observes that the human cells used in the experiment described in Exhibit D express both nestin (intermediate filament marker) and musashi 1, and mistakenly concludes that detection of both would have been required for one of ordinary skill in the art to obtain the cells for transplantation. As indicated in Exhibit D, nestin and musashi 1 are both phenotypic markers for neuroepithelial stem cells. The musashi 1 marker was more recently identified than nestin and, hence, merely confirms the neural epithelial status of the cells, which was already shown by nestin expression. Appellants submitted the Kawaguchi *et al.* (*Molecular and Cellular Neuroscience*, 2001, 17:259-273) and Sakakibara *et al.* (*PNAS*, 2002, 99(23):15194-15199) publications to the Patent Office with their Response under 37 C.F.R. §1.116 dated October 21, 2004. The Examiner considered and commented on the Kawaguchi *et al.* and Sakakibara *et al.* publications in the Advisory Action dated December 21, 2004. The Sakakibara *et al.* publication indicates that musashi 1 is a binding protein that is expressed in neural precursor cells, and which can be used as one marker to identify precursor cells. As indicated in the abstract of the Sakakibara *et al.* publication, the musashi family of proteins are evolutionarily conserved across species. In mammals, musashi 1 and musashi 2 are strongly co-expressed in neural precursor cells, including CNS stem cells. Likewise, the Kawaguchi *et al.* publication demonstrates that nestin is another marker that is expressed in neural precursor cells. Use of musashi 1 for this purpose of identifying neural precursor cells is thus equivalent to use of nestin. One of ordinary skill in the art would appreciate that musashi 1 expression does not represent an identifying characteristic of the cells that had to be discovered before the method of the invention could be carried out; demonstrating the expression of nestin by the cells, as taught in the specification, is sufficient. The mere fact that the human cells were further characterized and it was determined that the cells expressed musashi 1, in addition to nestin, should not disqualify the experimental results and



accompanying Declaration by Dr. Sinden as probative evidence in the determination of written description and enablement under 35 U.S.C. 112, first paragraph.

The Office Action dated May 25, 2004 states that “claiming a method of treating cognitive deficit using pluripotent, nestin-positive neuroepithelial cells in humans without defining the properties required to obtain human cells capable of treating a cognitive deficit ... is not in compliance with the description requirement ... It is not sufficient to define the method as requiring cells having particular biological properties, i.e., expressing nestin and pluripotent and capable of treating humans, because disclosure of no more than that, as in the instant case, is simply a wish to know the identity of human cells capable of restoring cognitive function” (emphasis added; page 8, lines 17-22, and page 9, lines 1-3 of the Office Action). This statement presupposes that pluripotency and nestin expression are insufficient to identify populations of cells capable of repairing damage as recited in the claims. The Examiner has provided no rationale for this position and has placed no evidence on the record to support the premise. The Examiner merely observes that the human nestin-positive pluripotent neuroepithelial cells described in Exhibit D are also musashi 1-positive, and mistakenly concludes, solely on that basis, that musashi 1 expression “may be” required:

The experimental results in the Declaration by Dr. Sinden remain unpersuasive because the cells used in the experiment were of a narrower scope than those described in the specification as originally filed and because musashi expression may be essential to obtain nestin-positive neural precursors cells with the desired function in humans (emphasis added; page 6, lines 11-15, of the Advisory Action dated December 21, 2004).

Certainly, the cells have still other characteristics that are not specified, but clearly they are not necessary to describe the cells. The Examiner confuses the possibility of further characterization with the necessity of doing so, and that is improper.

The Examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the original disclosure a description of the invention defined by the claims. The Examiner must have a reasonable basis to challenge the adequacy of the written description and must establish, by a preponderance of evidence, why a person skilled in the art would not recognize in an applicant’s disclosure a description of the invention defined by the claims. In rejecting a claim, an Examiner must set forth express

findings of fact, applying the necessary analysis, which support the conclusion that there is a lack of written description. This the Examiner has not done.

An applicant is not required to describe every detail of his invention. An applicant may show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics that provide evidence that the applicant was in possession of the claimed invention. *Enzo Biochem, Inc. v. Gene-Probe Inc.*, 285 F.3d 1013; 62 USPQ2d 1289 (Fed. Cir. 2002) and *Enzo Biochem, Inc. v. Gene-Probe Inc., on rehearing* 296 F.3d 1316; USPQ2d 1609 (Fed. Cir. 2002) *rehearing en banc denied*. Such characteristics can include (a) complete or partial structure of the claimed invention; (b) functional characteristics, provided there is a correlation between the function and structure of the claimed invention; (c) physical properties, and/or (d) chemical properties (See, for example, “Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112 para. 1 ‘Written Description’ Requirement.” 66 Fed. Reg. 1099, 1106 (January 5, 2001)). The disclosure of any combination of identifying characteristics that “distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species” is sufficient to comply with the written description requirement (“Guidelines for Examination of Patent Applications under the 35 U.S.C. 112 para. 1 ‘Written Description’ Requirement” at 1106). As indicated above, neuroepithelial cells can be screened for pluripotency *in vitro*, to verify their ability to differentiate upon transplantation, as taught at page 13, lines 17-28, and Example 4, at pages 20-21 of the ‘274 application. An applicant’s disclosure obligation varies according to the art to which the invention pertains. The currently pending claims are drawn to methods of using cells. In addition to pluripotency, the ‘274 application discloses that neuroepithelial cells suitable for transplantation are nestin-positive. Immuno-detection of cell-surface markers is an art-recognized technique for the characterization of cells and determination of cell fate. There is an art-recognized correlation between cell type, *e.g.*, as determined by the cell’s surface markers (the cell’s chemical “structure”), and the properties and functions exhibited by the cell. The ‘274 application describes the recited genus of human neuroepithelial cells sufficient to distinguish the recited cells from other cells, providing sufficient characteristics by which to identify human neuroepithelial cells that may be used in the claimed invention.

Appellants respectfully submit that the ‘274 application provides relevant identifying characteristics sufficient to describe the claimed invention in such full, clear, concise, and exact

terms that one of ordinary skill in the art would recognize that Appellants were in possession of the claimed invention. Accordingly, Appellants request that this rejection of the claims under 35 U.S.C. §112, first paragraph, be reversed.

Claims 57, 58, 60-62, 76-80, 83, and 84

In the event that the Board believes this rejection should be sustained on at least claims 64, 81, 82, 85, and 86, Appellants respectfully assert that it should certainly be overruled as to claims 57, 58, 60-62, 76-80, 83, and 84, which do not have the specific limitations that the transplanted cells are human cells or that the mammal is human. For the reasons described above, the pluripotent neuroepithelial cells described in the '274 application are representative of the class of cells recited in claims 57, 58, 60-62, 76-80, 83, and 84.

**B. Claims 57, 58, 81, 82, 85, and 86 do not constitute new matter, because Appellants' specification provides support for each element of the claims.**

Claims 57, 58, 81, 82, 85, and 86 stand rejected under 35 U.S.C. §112, first paragraph, in various combinations (argued separately herein), on the grounds that the following phrases or concepts represent new matter:

1. "a disorder associated with damage to, or loss of, brain cells in a mammal" in claims 57, 81, and 85;
2. "wherein said cells are immortal prior to said transplanting and differentiate after said transplanting" in claim 57;
3. "wherein said transplanting improves brain function of said mammal" in claims 57 and 81;
4. "a disorder associated with damage to, or loss of, brain cells in the hippocampus of said mammal" in claim 58;
5. "wherein said transplanting improves cognitive function of said mammal" in claims 57 and 81; and
6. treating a "human", as recited in claims 82 and 86.

Appellants respectfully submit that claims 57, 58, 81, 82, 85, and 86 do not constitute new matter. Support for the phrase "a disorder associated with damage to, or loss of, brain cells

in a mammal”, which is recited in claims 57, 81, and 85, can be found throughout the subject application, and particularly at page 1, lines 19-25, and page 5, lines 15-22, of the specification as originally filed. The specification states that when transplanted, “pluripotent neuroepithelial cells appear to respond to signals from the damaged or diseased brain by taking up a phenotype that is able to replace or compensate for functional deficits to which the damage or disease otherwise leads” and that “the phenotype of the differentiated cells may be the same as the phenotype of the damaged or lost cells, however, the differentiated cells may be of a different phenotype” and “the cells take up a phenotype that is capable of functionally integrating and compensating for the damaged or lost cells.” At page 2, lines 14-17, the specification states that “the treatment may be carried out on any mammal.” Thus, it is clear that the phrase “a disorder associated with damage to, or loss of, brain cells in a mammal”, found in claims 57, 81, and 85 is adequately supported.

Support for the phrase “wherein said cells are immortal prior to said transplanting and differentiate after said transplanting”, which is recited in claim 57, can be found, for example, at page 5, lines 10-15 and lines 32-36, and page 6, lines 1-10 and 16-25, of the specification as originally filed. At page 5, lines 10-15, the specification states that “when conditionally immortal pluripotent neuroepithelial cells are implanted into a damaged brain the cells differentiate into the correct form of cell required to repair the damage and the differentiated cells are able to form the appropriate connections required to improve function” and that “conditionally immortal cells are cells which are immortal under certain permissive conditions but are not immortal under nonpermissive conditions”. Thus, as explained at page 6, lines 6-10, of the specification, “if the conditions under which the cells are maintained are switched to nonpermissive conditions, the development of the cells is allowed to continue. If the correct conditions are provided the cells will continue to develop and will differentiate”. As stated at page 6, lines 16-25, of the specification, “conditionally immortal cells have the advantages of immortal cells in that they are “frozen” in the desired stage of development, are easily maintained and multiply well when under permissive conditions but they may be used in transplants as long as the environment into which they are transplanted has nonpermissive conditions.” Thus, it is clear that the phrase “wherein said cells are immortal prior to said transplanting and differentiate after said transplanting”, found in claim 57, is adequately supported.

Support for the phrase “wherein said transplanting improves brain function of said mammal”, which is recited in claims 57 and 81, can be found, for example, at page 8, lines 1-5, which states that the transplanted cells are able to “differentiate in response to the local microenvironment, into the necessary phenotype or phenotypes to improve or restore function.” At page 1, lines 18-23, the specification states that when transplanted into a damaged or diseased brain, “pluripotent neuroepithelial cells appear to respond to signals from the damaged or diseased brain by taking up a phenotype that is able to replace or compensate for functional deficits to which the damage or disease otherwise leads”. Furthermore, at page 8, lines 21-27, the specification states that, “preferably, the treatment will substantially correct a behavioral and/or psychological deficit ... [H]owever, treatment according to the present invention ... may lead to improvement in function without complete correction.” Page 10, lines 34-36, and page 11, lines 1-7, state

Thus it appears that the cell lines are capable of responding to damage-associated signals so as to differentiate into cells, of one or more types, that are able re-establish the necessary connections and restore the functions(s) discharged by the damaged tissue. It is this capacity that provides both a strategy and a material basis for transplant therapies with which to target a wide range of behavioral and psychological deficits consequent upon an equally wide range of forms of damage to the human brain....

Thus, it is clear that the phrase “wherein said transplanting improves brain function of said mammal”, found in claims 57 and 81, is adequately supported.

Support for the phrase “a disorder associated with damage to, or loss of, brain cells in the hippocampus of said mammal”, which is recited in claim 58, can be found, for example, at page 5, lines 15-22, page 13, lines 2-4, and Examples 5-9 at pages 22-29, of the specification as originally filed. At page 5, lines 15-22, the specification indicates that the implanted cells take up a phenotype that is capable of functionally integrating and compensating for damaged or lost cells. As described in Example 5 at page 22 of the specification, ischemic lesions were created in the CA1 area of the hippocampus of rats and restoration of spatial learning and memory was evaluated using the Morris water maze test. In Example 9, at pages 27-29, the specification describes assessment of post-mortem ischaemic brain damage, and indicates ischemia was present in the hippocampus of the animal model (see, for example, page 28, lines 1-2). As

indicated at page 9, lines 29-35, and page 10, lines 1-9, the lesion-and-behavior model used in Examples 5-9 uses a technique of four-vessel occlusion (4 VO), “causing relatively circumscribed and specific damage to the CA1 pyramidal cells of the dorsal hippocampus, along with a cognitive deficit...”. The Examiner also acknowledged that the ‘274 application teaches treatment of damage in the hippocampus. The Office Action dated May 25, 2004 states “the specification is limited to treating damage to hippocampal cells using hippocampal, pluripotent, nestin-positive, neuroepithelial cells” (page 14, lines 18-19; emphasis added); and “therefore, the claims should be limited to treating damage to hippocampal brain cells using hippocampal, pluripotent, nestin-positive, neuroepithelial cells” (page 15, lines 2-3; emphasis added). Thus, it is clear that the phrase “a disorder associated with damage to, or loss of, brain cells in the hippocampus of said mammal”, found in claim 58, is adequately supported.

The Advisory Action dated December 21, 2004 states that the phrase “wherein said transplanting improves cognitive function of said mammal”, as recited in claims 57 and 81, is new matter. Appellants submit that this aspect of the rejection appears inconsistent with the basis of the rejection offered at page 4 of the Advisory Action, which states “the specification does not support improving any ‘brain function’ as newly amended by adding neuroepithelial cells because the specification only taught using a model of cognitive function” (emphasis added). The Examiner also acknowledged that evaluation and/or improvement in cognitive function is taught in the specification elsewhere during prosecution; see, for example, page 7, lines 12-13, of the Office Action dated August 20, 2003; and page 6, line 15, page 12, lines 17-18, and page 15, lines 20-21, of the Office Action dated May 25, 2004. Page 15, lines 220-21 states “the specification taught using mouse, pluripotent, nestin-positive, hippocampal neuroepithelial cells to restore cognitive function in rats” (emphasis added). Thus, it is clear that the phrase “wherein said transplanting improves cognitive function of said mammal”, found in claims 57 and 81, is adequately supported.

Support for the phrase “wherein said transplanting improves cognitive function of said mammal”, recited in claims 57 and 81, can be found, for example, at page 8, lines 15-27, of the specification as originally filed, which states:

After treatment the progress of the patient may be monitored using behavioral and/or psychological tests and/or, if desired, tests which monitor brain activity in selected areas of the brain. For example, tests for cognitive function may be performed before and after transplantation.

Preferably, treatment will substantially correct a behavioral and/or psychological deficit. However, that may not always be possible. Treatment according to the present invention and with the cells, medicaments and pharmaceutical preparations of the invention, may lead to improvement in function without complete correction. Such improvement will be worthwhile and of value. (emphasis added)

Furthermore, at page 9, lines 29-35, and page 10, lines 1-9, the '274 application teaches that the lesion-and-behavior model used in Examples 5-9 of the application causes a cognitive deficit due to loss of blood supply to the brain. The Morris water maze test was used to evaluate improvement in cognitive function. Thus, it is clear that the phrase "wherein said transplanting improves cognitive function of said mammal", found in claims 57 and 81, is adequately supported.

Support for the treatment of humans, as recited in claims 82 and 86, can be found throughout the specification, where it is clear that treatment of humans was contemplated. For example, at page 2, lines 14-17, the specification as originally filed indicates that "treatment may be carried out on any mammal but the present invention is especially concerned with the treatment of humans, especially treatment with human cells, and with human cells and cell lines" (emphasis added). At page 4, lines 1-3, the specification as originally filed indicates "a further aspect of the invention provides for conditionally immortal, pluripotent, neuroepithelial cells for therapeutic use, especially in humans" (emphasis added). At page 7, lines 8-10, the specification indicates "the cells used in the treatment of humans should preferably be derived from human cells to reduce problems with immune rejection" (emphasis added). Thus, it is clear that treatment of humans, recited in claims 57 and 81, is adequately supported.

Contrary to the statements at page 4 of the Advisory Action, Appellants respectfully submit that the specification as a whole does support using neuroepithelial cells other than hippocampal neuroepithelial cells. At page 13, lines 5-7, the specification teaches that "the part of the fetal brain from which the neuroepithelial cells are taken and the precise time (stage and development) may vary". Furthermore, as indicated at page 12, lines 33-36, and page 13, lines 1-4, uses of cells according to the invention are not limited to repair of the particular type of damage modeled in Examples 6, 7, and 8; rather, "transplantation into any area of the brain is envisaged with consequent improvement in function". Examples of diseases or conditions that

result in behavioral and/or psychological deficits that may be treated in accordance with the present invention are set forth at page 3, lines 1-14, of the specification as originally filed.

The subject matter of a claim need not be described literally (*i.e.*, using the same terms or *in haec verba*) in order for the disclosure to satisfy the written description requirement. “It is not necessary that the application describe the claim limitations exactly,...but only so clearly that persons of ordinary skill in the art will recognize from the disclosure that appellants invented processes including those limitations”. *In re Wertheim*, 541 F.2d 257, 262; 191 USPQ 90, 96 (C.C.P.A. 1976). “*Ipsis verbis* disclosure is not necessary to satisfy the written description requirement of section 112. Instead, the disclosure need only reasonably convey to persons skilled in the art that the inventor had possession of the subject matter in question.” *Fujikawa v. Wattanasin*, 93 F.3d 1559; 39 USPQ2d 1895, 1904 (Fed. Cir. 1996), quoting from *In re Edwards*, 568 F.2d 1349, 1351-52; 196 USPQ 465, 467 (CCPA 1978). Appellants respectfully submit that the specification provides adequate support for the claimed subject matter, describing the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed subject matter, which is all that is required to satisfy the written description requirement of 35 U.S.C. §112, first paragraph. The claimed subject matter does not represent new matter. Accordingly, Appellants request that this rejection of the claims under 35 U.S.C. §112, first paragraph, be reversed.

**C. The specification enables a method for treating a disorder associated with damage to, or loss of, brain cells in a mammal by intracerebrally transplanting pluripotent, nestin-positive, neuroepithelial cells into the brain of the mammal, wherein the cells have been genetically modified to be conditionally immortal, wherein the cells are immortal prior to transplantation and differentiate after transplantation, and wherein transplantation improves brain function of the mammal, as recited in claims 57, 58, 60-62, and 76-86.**

**1. Appellants’ specification, which broadly teaches that pluripotent, nestin-positive neuroepithelial cells may be obtained from various areas of the brain and intracerebrally transplanted to treat a disorder associated with damage to, or loss of, brain cells, and exemplifies using mouse hippocampal**



**pluripotent, nestin-positive neuroepithelial cells to treat a cognitive deficit associated with damage to, or loss of brain cells in the hippocampus, enables claims that are not limited to transplantation of mouse hippocampal cells to treat a cognitive deficit associated with damage to, or loss of hippocampal cells.**

Appellants submit that, given the benefit of the specification's disclosure, a person of ordinary skill in the art could readily identify and use nestin-positive, pluripotent neuroepithelial cells as recited in the claims. Evidence of the ability to use a variety of pluripotent, nestin-positive neuroepithelial cells to treat various intracerebral tissues is provided within Exhibit D, which accompanied the Sinden Declaration, described above. Exhibit D describes an experiment carried out using human nestin-positive pluripotent neuroepithelial cells derived from the human fetal cortex, to treat damage associated with the basal forebrain. These cells were genetically modified to be conditionally immortal, in accordance with Appellants' teachings, and as recited in the currently pending claims. As indicated above, Appellants respectfully submit that the teachings of the specification and Exhibit D are consistent, and the human nestin-positive neuroepithelial cells described in Exhibit D correlate with the teachings of the subject specification as originally filed. As indicated at page 13, lines 5-7 of the '274 application, the region of the brain from which neuroepithelial cells are obtained and the precise time (stage and development) they are obtained may vary.

The Sinden *et al.* publication (*Neuroscience*, 81:599-608, 1997) was cited in the Office Action dated May 25, 2004 and previous Actions as suggesting that CA1 cells derived from the hippocampus must be used to repair damaged CA1 hippocampal tissue. However, the cited portion of the Sinden *et al.* (1997) reference is merely characterizing the prior art. As indicated in the Sinden Declaration, "the statement referred to by the Reviewer within the Sinden *et al.* publication (of which I am the first author), is made with respect to a previous study that used primary cells that were mature, differentiated or committed CA1 cells, and not the conditionally immortal, pluripotent, nestin-positive, neuroepithelial cells that are used in the method of our invention" (emphasis added).

As indicated in the Sinden Declaration, "provided the neuroepithelial cells are nestin-positive and retain the ability to differentiate into the specified phenotypes in response to

environmental signals, they are appropriate for use in the present invention.” The Scheffler *et al.* publication does not provide any reason to doubt that one of ordinary skill in the art, having the benefit of the specification’s disclosure, can determine what is, and what is not, an appropriate pluripotent neuroepithelial cell for use in the subject invention.

While Appellants acknowledge that some experimentation and screening may be required to isolate human, pluripotent, nestin-positive neuroepithelial cells, the court in *In re Wands* has stated

Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is ‘undue’ not ‘experimentation’.

The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731; 8 USPQ2d 1400 (Fed. Cir. 1988).

The cells recited in the claimed methods are positive for the progenitor cell marker, nestin. As indicated in the Sinden Declaration, “nestin-positive cells can be readily identified using immunocytochemistry,” as described at pages 20 and 21 of the subject patent application, or by other techniques known to those of ordinary skill in the art. Furthermore, Example 4 of the ‘274 application describes an *in vitro* screening method for determining the pluripotency of the cells *in vivo* (see page 13, lines 23-26). The experimentation and screening required to obtain the necessary pluripotent, nestin-positive neuroepithelial cells are standard and routine in the art. Thus, Appellants respectfully submit that the ‘274 application provides adequate guidance for the skilled person to identify and use appropriate cells, without resort to undue experimentation.

**2. Appellants’ specification, which broadly teaches that conditionally immortal pluripotent, nestin-positive neuroepithelial cells may be transplanted to any portion of the brain, and exemplifies transplanting conditionally immortal pluripotent, nestin-positive neuroepithelial cells to the**

**hippocampus, enables claims that are not limited to transplantation to the hippocampus.**

The Scheffler *et al.* publication (*Trends in Neurosci.*, Vol. 22, pg. 348-357, 1999) has been cited in the Office Action dated May 25, 2004 (page 17) and earlier Actions as showing that it was “unpredictable” how to target particular areas of the brain when transplanting neural cells. However, the relevant passage in the Scheffler *et al.* publication refers to a study that used transplanted post-natal and adult neurons (therefore, differentiated neurons), and did not relate to the transplantation of conditionally immortal pluripotent neuroepithelial cells. As indicated in the Sinden Declaration, “one of the great advantages of the present invention is that it is not necessary to target particular areas of the brain to correct cell damage.” This is emphasized at various points throughout the ‘274 application, as well (see, for example, page 5, lines 10-31). As Dr. Sinden explains in his Declaration,

previously, it was thought that to treat damage in a developed postnatal or adult brain, it was necessary to use tissue/cells derived from the same area as that damaged. Importantly, prior to our invention, even if the cells to be transplanted were taken from a fetus, ... the cells would typically be committed to a particular phenotype. Moreover, prior to our work, there was no selection of nestin-positive, pluripotent cells, or genetic modification of the cells to confer conditional immortality such that the cells would be immortal prior to transplantation but differentiate subsequent to transplantation (page 2 of the Sinden Declaration).

The inventors realized that, “surprisingly, transplanting cells that were selected to retain a nestin-positive, pluripotent, conditionally immortal phenotype resulted in the repair of damage, and this was independent of the site of damage”, as indicated in Dr. Sinden’s Declaration. Accordingly, using the cells of the claimed invention permits treatment at different sites of damage with a single cell line, which is selected on the basis of its nestin-positive, pluripotent characteristics, and is genetically modified to be conditionally immortal.

In contrast to the observations made in the Scheffler *et al.* publication, which involved the use of differentiated post-natal and adult neurons, as stated above, Appellants have shown that the targeting of cells is not necessary using the pluripotent cells of the subject invention. The subject specification teaches that the pluripotent cells migrate to areas of damage after transplantation, and become integrated in the damaged areas, effecting repair. The transplanted

cells migrate as necessary, populating the damaged areas of the brain, which overcomes the difficulties highlighted by the Scheffler *et al.* publication relating to the use of differentiated neurons in transplantation. As explained by Dr. Sinden in his Declaration, “this ability of the cells to migrate (which we were the first to observe) is an inherent feature of the cells; therefore, the difficulties identified in the Scheffler *et al.* publication will not be experienced when using nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal” (page 2 of the Sinden Declaration).

It is well settled patent law that an applicant’s statements must be accepted as true unless the Patent Office can provide evidence to doubt the truth of those statements. *In re Marzocchi*, 439 F.2d 220; 169 USPQ 367 (CCPA 1971). The Examiner has not provided acceptable reasoning for doubting the statements within the specification or the Declaration by Dr. Sinden. The record is replete with evidence supporting the truth of the ‘274 application’s teachings that the cells of the invention migrate to areas of damage after transplantation, become integrated within the damaged brain, and achieve repair.

At page 17 of the Office Action dated May 25, 2004, the Examiner states “a mere statement that the cells do not require targeting particular areas of the brain without evidence or scientific reasoning is inadequate to overcome the rejection”. Appellants assert that more than “a mere statement” has been made of record in support of the ability of the recited cells to migrate to different areas of the brain, integrate, and effect repair. Submitted with the Sinden Declaration as Exhibit B was a copy of U.S. Patent Application Publication No. 2002/0037277 (now U.S. Patent No. 6,569,421; referred to herein as the ‘277 publication). The example at pages 2-5 of the published application clearly demonstrates migration of the cells, and also shows that cells from one region of the brain (hippocampal region) can repair damage to a different area of the brain, such as cortex and basal ganglia, thus proving the truth of the subject ‘274 application’s teachings.

The cells utilized in the example of the ‘277 publication were nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal, as recited in the currently pending claims. The Office Action dated May 25, 2004 indicates that “no evidence can be found that the cells in Exhibit B were prepared the same as those described in the specification” (page 18 of the Office Action). Appellants point to paragraph 0030 at column 2 of the ‘277 publication, which states “conditionally immortal pluripotent neuroepithelial cells

from the MHP36 clonal cell line were prepared as disclosed in WO-A-97/10329” (emphasis added), which is the published PCT application (PCT/GB96/02251) to which the subject ‘274 application claims priority. The MHP36 clonal cell line is used in the Examples in the ‘274 application (see, for example, page 8, lines 35-36; page 9, lines 1-29; page 10, lines 23-36; page 11, lines 1-9; pages 15-16; and pages 19-29 of the ‘274 application).

As indicated in the Sinden Declaration, regarding the ‘277 publication, “compelling evidence of extensive migration is presented at page 4, paragraph 0047, which indicates that contralaterally grafted cells ‘migrated across the midline to the opposite side of the brain (emphasis added).” The Office Action dated May 25, 2004 indicates that page 4, paragraph 0047, of the ‘277 publication “merely discusses migration and does not state adequate numbers of cells migrated to the site of tissue damage such that the cognitive deficit was treated” (page 18 of the Office Action). The ‘277 publication indicates that migration of implanted cells was extensive (see paragraphs 0047 – 0049). The issue of the number of migrating cells in the experiment is of limited relevance, since functional recovery was confirmed. Appellants point to pages 4 and 5, paragraphs 0050 – 0053, of the ‘277 publication. The stated purpose of the experiments was to determine whether grafts of MHP36 cells, from a conditionally immortalized clonal line, would promote functional recovery from stroke damage when placed in the intact hemisphere contra-lateral to the infarct cavity (see paragraph 0050 at page 4 of the ‘277 publication). As indicated in paragraphs 0050 and 0051 of the ‘277 publication, “the findings indicate that both sensorimotor and motor asymmetries were normalized in rats with grafts initially sited in the intact hemisphere...The evidence for recovery of sensorimotor and motor functions is robust, because improvements were seen over an extended time period.”

Appellants note that the currently pending claims recite that transplanting the cells improves brain function (*e.g.*, cognitive function) in the mammal. Thus, it would be understood by those skilled in the art that the number of cells transplanted would be an amount effective to achieve the recited improvement in function. Guidance regarding the number of cells to be transplanted is provided page 8, lines 28-34, of the ‘274 application as originally filed.

The Snyder *et al.* patent (U.S. Patent No. 6,528,306), of record, describes the migratory properties of neural stem cells and demonstrates that the cells can be maintained in culture in an undifferentiated state, with differentiation occurring upon transplantation. The Snyder *et al.* patent was not publicly available at the priority date of the subject application; however, the

patent demonstrates that the '274 application contained all the information necessary for the skilled person to carry out the invention. The background section of the Snyder *et al.* patent cites several early scientific papers reporting the behavior exhibited by neural stem cells upon transplantation. For example, at column 1, lines 44-56, the Snyder *et al.* patent indicates that neural stem cells are extremely plastic and can migrate and differentiate "in a temporally and regionally appropriate manner ..., responding similarly to local microenvironmental cues for their phenotypic determination and appropriately differentiating into diverse neuronal and glial cell types." Furthermore, at column 1, lines 40-43, the Snyder *et al.* patent cites several early publications as demonstrating the behavior of pluripotent neural stem cells following transplantation, including their ability to interact with host cells and differentiate appropriately.

Accordingly, Appellants respectfully submit that the scope of enablement provided by the subject '274 application bears a reasonable correlation to the scope of the claims; the '274 application enables claims that are not limited to transplantation to the hippocampus.

**3. Appellants' specification, which broadly teaches that conditionally immortal pluripotent, nestin-positive neuroepithelial cells may be intracerebrally transplanted into a mammal, such as a human, to treat a disorder associated with damage to, or loss of, brain cells, and exemplifies transplanting conditionally immortal pluripotent, nestin-positive neuroepithelial cells to a rat model, enables claims that are not limited to transplantation to a rat.**

Claims 57, 58, 60-62, and 76-84 recite a method for treating a disorder associated with damage to, or loss of, brain cells in a mammal. Claim 85 recites a method for treating a cognitive deficit caused by damage to the hippocampus of a mammal. Claims 64, 82, and 86 specify that the mammal is human.

In the Office Action dated May 25, 2004, the Examiner states "any new enablement rejections below have been made because of the increased breadth of the claim 57 and the breadth of new claims 81 and 85 ... Applicants claim a method of treating a human using neural stem cells expressing nestin and capable of self-renewal, but have not linked cells having such a

phenotype to the ability to treat cognitive deficits in humans” (page 12, lines 9-13, of the Office Action).

Appellants are again perplexed by the apparent inconsistency in the Examiner’s maintenance of this aspect of the rejection. In the Office Action dated December 23, 2002, claims 57-67 were rejected under 35 U.S.C. §112, first paragraph, as non-enabled. Claim 57 recited “a method of treating a cognitive deficit in a mammal, said method comprising intracerebrally transplanting pluripotent, nestin-positive, neuroepithelial cells into said mammal, wherein said cells have been genetically modified to be conditionally immortal, wherein said cells are immortal prior to said transplantation but differentiate after said transplantation, and wherein said transplantation improves cognitive function in said mammal.” At page 7 of the Office Action dated December 23, 2002, the Examiner states “the rejection regarding the breadth of ‘animal’ has been withdrawn because the claims have been amended to ‘mammal’ and because the rats having a lesion in the hippocampus used throughout the specification were treated with mouse cells.”

Appellants respectfully assert that the animal model exemplified in Examples 5-9 of the ‘274 application is predictive of the applicability of the claimed invention to other mammals, including humans. Furthermore, experimental data has been made of record in the ‘274 application that confirms the efficacy of the conditionally immortal nestin-positive neuroepithelial cells in treating primates, including marmosets and humans. In experiments described by Virley *et al.* (*Brain*, 1999, 122:101-115), which accompanied the Sinden Declaration as Exhibit C, pluripotent MHP36 cells (mouse cells exemplified in the ‘274 application) were transplanted to the brains of marmosets (a primate). As indicated at pages 3-4 of Dr. Sinden’s Declaration, “these cells performed as well within the marmoset brain as the marmoset fetal allografts, which suggests a low immune response provocation and confirms the applicability of our invention to treat primates, including humans, with a reasonable expectation of success” (see paragraph bridging pages 111-112 of the Virley *et al.* publication).

Experimental data demonstrating transplantation of human cells in a rat model of brain damage has also been made of record. The human cells used in the experiment described in Exhibit D express both nestin (intermediate filament marker) and musashi 1. As indicated in Exhibit D, nestin and musashi 1 are both phenotypic markers for neuroepithelial stem cells. The musashi 1 marker was more recently identified than nestin and, hence, merely confirms the neural

epithelial status of the cells, as also determined by nestin expression. As indicated above with regard to the rejection for lack of written description, the Kawaguchi *et al.* and Sakakibara *et al.* publications are of record. The Sakakibara *et al.* publication indicates that musashi is a binding protein that is expressed in neural precursor cells, which can be used to identify precursor cells. As indicated in the abstract of the Sakakibara *et al.* publication, the musashi family of proteins are evolutionarily conserved across species. In mammals, musashi 1 and musashi 2 are strongly co-expressed in neural precursor cells, including CNS stem cells. Likewise, the Kawaguchi *et al.* publication demonstrates that nestin is expressed in neural precursor cells. Use of musashi 1 is equivalent to use of nestin for identifying neural precursor cells. Thus, detection of musashi 1 expression, in addition to detection of nestin expression, is not “essential” or “required”. One of ordinary skill in the art would appreciate that musashi 1 expression does not represent a characterizing feature of the cells that had to be discovered before the invention could be carried out; demonstrating the expression of nestin by the cells, as taught in the specification, is sufficient. The mere fact that the human cells were further characterized and it was determined that the cells expressed musashi 1, in addition to nestin, should not disqualify the experimental results and accompanying Declaration by Dr. Sinden as probative evidence in the determination of enablement under 35 U.S.C. 112, first paragraph.

Thus, the subject ‘274 application describes a working example involving the transplantation of mouse cells in a rat model of brain damage characterized by damage to, or loss of, brain cells (Examples 5-9). Appellants have also submitted experimental data describing: (i) transplantation of mouse cells in a primate (marmoset) model of brain damage characterized by damage to, or loss of, brain cells (the Virley *et al.* publication; Exhibit C); and (ii) transplantation of human cells in a rat model of brain damage characterized by damage to, or loss of, brain cells (Exhibit D). The record is replete with evidence of the versatility of the claimed methods to treat the recited genus of mammals, including humans. Accordingly, Appellants respectfully submit that the scope of enablement provided by the subject ‘274 application bears a reasonable correlation to the scope of the claims.

**4. Appellants’ specification, which broadly teaches that pluripotent, nestin-positive neuroepithelial cells may be genetically modified to be conditionally immortal, such that the cells are immortal prior to**



**transplantation and differentiate after transplantation, and exemplifies transduction with a temperature-sensitive simian virus 40 large T antigen under the control of an interferon-inducible H-2K<sup>b</sup> promoter, enables claims that are not limited to the temperature-sensitive simian virus 40 large T antigen under the control of the interferon-inducible H-2K<sup>b</sup> promoter.**

The Office Action dated May 25, 2004 indicates that the '274 application does not provide sufficient guidance for conferring conditional immortality to cells (for example, via the temperature sensitive oncogene (tsA58)). As taught in the '274 application, such conditionally immortal cells can be readily prepared by transduction of an oncogene into a cell (see, for example, page 6 of the specification). As taught at page 7, lines 1-7, of the '274 application, the use of non-human transgenic animals is but one method for obtaining conditionally immortalized cells. Conditional immortality is described on page 5, last paragraph, and pages 6 and 7 of the '274 application, and it is clear that the cells remain immortal (undifferentiated and continuously dividing) under one set of conditions, but can be induced to mature and differentiate (losing immortality) by a change in conditions. Page 12, lines 23-31, of the '274 application states:

It should be understood that although the experiments described in the Examples below have been carried out using the ts SV40 large T antigen gene to confer conditional immortality on the cells, any other gene which is capable of causing conditional immortality may be used. Such genes may be constructed from known oncogenes. For example, a conditionally immortal gene has been constructed from the c-myc oncogene and is described by Hoshimai *et al.*, 1996.

The Frederiksen *et al.* publication (*Neuron*, 1998, Vol. 1, 439-448) and Jat *et al.* publication (*Proc. Natl. Acad. Sci. USA*, 1991, 88:5096-5100), of record, which accompanied the Amendments submitted on September 30, 2002 and March 20, 2003, respectively, show that methods for achieving conditional immortality using, for example, the temperature-sensitive SV40 oncogene, were known in the art even in 1988. The background section of the Snyder *et al.* patent also highlights methods (both epigenetic and genetic) for immortalizing cells that are not dependent on the large T antigen temperature-sensitive oncogene (see column 1, lines 31-43 of the Snyder *et al.* patent). Therefore, Appellants respectfully assert that the specification fully enables the use of pluripotent, nestin-positive neuroepithelial cells, including human cells, which have been genetically modified to be conditionally immortal, as recited in the claims.

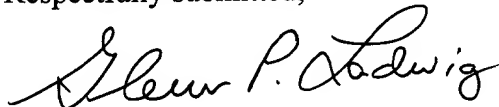
To exemplify their invention, Appellants used a widely applicable conditional immortalization method known to those skilled in the art as of the filing date. Although the temperature-sensitive simian virus 40 large T antigen under the control of an interferon-inducible H-2K<sup>b</sup> promoter was the genetic construct used by Appellants in demonstrating the truth of their teachings that nestin-positive, pluripotent neuroepithelial cells genetically modified to be conditionally immortal could be intracerebrally transplanted to treat a disorder associated with damage to, or loss of, brain cells, they clearly and unambiguously taught that additional methods of conditional immortalization could be used. They clearly indicated that the method of conditional immortalization is not essential to the invention. Rather, any gene known to effectively cause conditional immortality can be used in accord with Appellants' teachings. It is inequitable that Appellants' claims should be so limited that as newer immortalization technology becomes available, it provides a way around the claimed invention, which is not restricted to one type of conditional immortalization protocol over another.

Appellants respectfully submit that a person skilled in the art, having the benefit of the specification's disclosure, could readily make and use the claimed invention without resort to undue experimentation. Accordingly, Appellants request that this rejection of the claims under 35 U.S.C. §112, first paragraph, be reversed.

## CONCLUSION

In view of the foregoing, Appellants urge that the Board overrule the outstanding rejections under 35 U.S.C. §112, first paragraph, and that this application be passed to issuance.

Respectfully submitted,



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Attachments: Appendix A: Claims Appendix  
Appendix B: Evidence Appendix



APPENDIX A

Currently Pending Claims

(Claims 1-48, 59, and 68-75 cancelled in Amendment dated February 20, 2004)

(Claims 49-56 cancelled in Amendment dated September 30, 2002)

(Claims 63 and 65-67 cancelled in Amendment dated March 20, 2003)

Claim 57. A method for treating a disorder associated with damage to, or loss of, brain cells in a mammal, said method comprising intracerebrally transplanting pluripotent, nestin-positive, neuroepithelial cells into the brain of said mammal, wherein said cells have been genetically modified to be conditionally immortal, wherein said cells are immortal prior to said transplanting and differentiate after said transplanting, and wherein said transplanting improves brain function of said mammal.

Claim 58. The method of claim 57, wherein the disorder is associated with damage to, or loss of, brain cells in the hippocampus of said mammal.

Claim 60. The method of claim 57, wherein said pluripotent, nestin-positive, neuroepithelial cells are cells of a clonal cell line.

Claim 61. The method of claim 57, wherein said method further comprises culturing said pluripotent, nestin-positive, neuroepithelial cells in serum-free medium prior to said transplanting.

Claim 62. The method of claim 57, wherein the disorder is the result of hypoxia.

Claim 64. The method of claim 57, wherein said mammal is a human.

Claim 76. The method of claim 57, wherein the disorder comprises a cognitive deficit, and wherein the brain function comprises cognitive function.

Claim 77. The method of claim 57, wherein the genetic modification comprises transduction with a temperature-sensitive oncogene.

Claim 78. The method of claim 57, wherein the genetic modification comprises transduction with a temperature-sensitive simian virus 40 large T antigen gene.

Claim 79. The method of claim 57, wherein the genetic modification comprises transduction with a temperature-sensitive simian virus 40 large T antigen gene under the control of an interferon-inducible H-2K<sup>b</sup> promoter.

Claim 80. The method of claim 57, wherein said cells are immortal at 33° C and differentiate at 39° C.

Claim 81. A method for treating a disorder associated with damage to, or loss of, brain cells in a mammal, said method comprising intracerebrally transplanting human pluripotent, nestin-positive neuroepithelial cells into the brain of said mammal, wherein said human pluripotent, nestin-positive neuroepithelial cells comprise a temperature-sensitive simian virus 40 large T antigen gene, and wherein said transplanting improves brain function of said mammal.

Claim 82. The method of claim 81, wherein said mammal is human.

Claim 83. The method of claim 81, wherein said cells are immortal at 33° C and differentiate at 39° C.

Claim 84. The method of claim 81, wherein said temperature-sensitive simian virus 40 large T antigen gene is under the control of an interferon-inducible H-2K<sup>b</sup> promoter.

Claim 85. A method for treating a cognitive deficit caused by damage to the hippocampus of a mammal, said method comprising intracerebrally transplanting human pluripotent, nestin-positive, hippocampal neuroepithelial cells into said hippocampus of said

mammal, wherein said human pluripotent, nestin-positive, hippocampal neuroepithelial cells comprise a temperature-sensitive simian virus 40 large T antigen gene under the control of an interferon-inducible H-2K<sup>b</sup> promoter, and wherein said transplanting improves cognitive function in said mammal.

Claim 86. The method of claim 85, wherein said mammal is human.



## **Appendix B**

1. Declaration of Dr. John Sinden under 37 C.F.R. §1.132, including Exhibits A-D, of record, which accompanied the Amendment submitted on September 30, 2002, and which were entered into the record in the Office Action dated December 23, 2003.
2. Copy of published article by Kawaguchi *et al.* (*Molecular and Cellular Neuroscience*, 2001, 17:259-273), which accompanied the Response submitted on October 21, 2004, and was entered into the record in the Advisory Action dated December 21, 2004.
3. Copy of published article by Sakakibara *et al.* (*PNAS*, 2002, 99(23):15194-15199), which accompanied the Response submitted on October 21, 2004, and was entered into the record in the Advisory Action dated December 21, 2004.
4. Copy of U.S. Patent No. 6,528,306 (Snyder *et al.*), which accompanied the Response submitted on February 20, 2004, and was entered into the record in the Office Action dated May 25, 2004.
5. Copy of published article by Jat *et al.* (*Proc. Natl. Acad. Sci. USA*, 1991, 88:5096-5100), which accompanied the Response submitted on March 20, 2003, and was entered into the record in the Office Action dated May 25, 2004.
6. Copy of published article by Frederiksen *et al.* (*Neuron.*, 1988, 1:439-448), which accompanied the Response submitted on September 30, 2002, and was entered into the record in the Office Action dated May 25, 2004.

Patent Application  
Docket No. GJE-21D2  
Serial No. 09/760,274

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner : Michael C. Wilson  
Art Unit : 1632  
Applicant : John Sinden, Jeffrey A. Gray, Helen Hodges, Timothy Kershaw,  
Fiza Rashid-Doubell  
Serial No. : 09/760,274  
Filed : January 12, 2001  
For : Neural Transplantation Using Pluripotent Neuroepithelial Cells

Assistant Commissioner for Patents  
Washington, D.C. 20231

DECLARATION OF JOHN SINDEN, Ph.D., UNDER 37 C.F.R. § 1.132

Sir:

I, John Sinden, Ph.D., of ReNeuron Limited, hereby declare:

THAT, my *curriculum vitae* is attached hereto as Exhibit A;

THAT, I am a named inventor on the above-referenced patent application;

THAT, I have read and understood the specification and claims of the subject application and the Office Action dated May 31, 2002;

AND, being thus duly qualified, do further declare:

1. Our invention is based on the surprising discovery that conditionally immortalized, nestin-positive, pluripotent neuroepithelial cells, when transplanted into a damaged brain, are capable of differentiating into the phenotype of the damaged part of the brain, thereby repairing the damage and restoring function that has been lost as a result of the damage. Furthermore, it has been found that these cells have the property of migrating from the site of application to the site of damage. Further work (for which evidence is now provided) confirms these initial observations, and provides evidence that a wide variety of human brain damage-related disorders are capable of treatment by using nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal.



2. Previously, it was thought that to treat damage in a developed postnatal or adult brain, it was necessary to use tissue/cells derived from the same area as that damaged. Importantly, prior to our invention, even if the cells to be transplanted were taken from a fetus, such as those described in the Netto *et al.* publication, the cells would typically be committed to a particular phenotype. Moreover, prior to our work, there was no selection of nestin-positive, pluripotent cells, or genetic modification of the cells to confer conditionally immortality such that the cells would be immortal prior to transplantation but differentiate subsequent to transplantation. We have realized that, surprisingly, transplanting cells that were selected to retain a nestin-positive, pluripotent, conditionally immortal phenotype resulted in the repair of damage, and this was independent of the site of damage.

3. In contrast to the observations made in the Scheffler *et al.* publication (*Brain and Bone Marrow*, 1999, 11:348-357), which is cited by the Reviewer in the Office Action, one of the great advantages of the present invention is that it is not necessary to target particular areas of the brain to correct cell damage. The cells used in our invention migrate to areas of damage after transplantation and become integrated in the damaged areas, effecting repair. This ability of the cells to migrate (which we were the first observe) is an inherent feature of the cells; therefore, the difficulties identified in the Scheffler *et al.* publication will not be experienced when using nestin-positive, pluripotent neuroepithelial cells that have been genetically modified to be conditionally immortal.

4. The Reviewer cites the Sinden *et al.* (1997) publication as suggesting that hippocampal CA1 cells must be used to repair CA1 tissue. However, the statement referred to by the Reviewer within the Sinden *et al.* publication (of which I am the first author), is made with respect to a previous study that used primary cells that were mature, differentiated or committed CA1 cells, and not the conditionally immortal, pluripotent, nestin-positive neuroepithelial cells that are used in the method of our invention. Provided the neuroepithelial cells are nestin-positive and retain the ability to differentiate into the specified phenotypes in response to environmental signals, they are appropriate

for use in the present invention. Nestin-positive cells can be readily identified using immunocytochemistry, for example.

5. The ability of conditionally immortal nestin-positive, pluripotent neuroepithelial cells to migrate is readily apparent in U.S. Patent Application Publication No. 2002/0037277 (hereinafter the '277 publication), which is attached as Exhibit B. The example at pages 2-5 of the '277 publication describes implantation of conditionally immortal, nestin-positive, pluripotent neuroepithelial cells into the unilaterally damaged brains of rats. The cells were implanted either ipsilaterally or contralaterally to the hemisphere containing the infarcted area, resulting in improved function. Compelling evidence of extensive migration is presented at page 4, paragraph 0047, which indicates that contralaterally grafted cells "migrated across the midline to the opposite side of the brain" (emphasis added). Certainly, the experiment shows that cells from one anatomical region of the brain (hippocampal region) can repair damage to a different anatomical region of the brain, such as cortex and basal ganglia.

6. The Reviewer refers to Sanberg *et al.* as teaching that immunosuppressive agents used for xenotransplanted cells may preclude any therapeutic benefit in humans because of the health risks associated with immunosuppression. However, in all forms of transplantation therapy, *e.g.*, liver, heart, *etc.*, immuno-suppressive agents are virtually always included as part of the treatment regimen to help avoid rejection. Also submitted herewith as Exhibit C is the Virley *et al.* publication (*Brain*, 1999, 122:101-115), which supports the efficacy of transplanting conditionally immortal nestin-positive, pluripotent cells into the damaged mammalian brain to restore function caused by cell damage or cell loss. The Virley *et al.* publication, of which I am a co-author, describes the implantation of pluripotent MHP36 cells into the bilaterally lesioned brains of primates (marmosets), resulting in improved function. These MHP36 cells, which are mouse cells, are also exemplified in our patent application. These cells performed as well within the marmoset brain as the marmoset fetal allografts, which suggests a low immune response provocation and confirms the applicability of

our invention to treat primates, in general, including humans, with a reasonable expectation of success.

7. The Morris water maze test was developed in the 1980s and has become the method of choice for assessment of spatial learning and memory in rodents. The test is also accepted by those skilled in the art as generally predictive of long-term neurological and behavioral outcome in humans and is routinely utilized in the development of repair strategies for human neuro-degenerative disorders. This is evidenced by the enormous number of publications in the field in which this model is utilized. A survey of the literature shows a general consensus that the water maze test is useful in assessing cognitive function (e.g., spatial learning and memory) within the context of a variety of etiologies (e.g., aging, neonatal stress, lesion or ischemic damage to cortex, striatum, or hippocampus) and treatments (Morris R.G.M. "Spatial localisation does not require the presence of local cues" *Learn Motiv.*, 12: 239-260, 1981; Rapp. P.R. *et al.* "An evaluation of spatial information processing in aged rats" *Behav. Neuroscience*, 101:3-12, 1987; Di Mattia R. and Kesner P. "Spatial cognitive maps: differential role of parietal cortex and hippocampal formation" *Behav. Neuroscience*, 102:471-480, 1988; Stewart C.A. *et al.* The water maze. In *Behavioural Neuroscience: a practical approach*, Rickwood D and Hames H.D. (eds), Oxford. OUP, pp106-122, 1993; Propoli P. *et al.* Behavioural and electrophysiological correlates of the quinolinic acid lesion model of Huntington's Disease in rats, 1994; Hodges H. Testing for spatial brain dysfunction in animals. In: *Handbook of Spatial Learning*, eds N. Foreman and R. Gillett, Chapter 15, 1998; McIlwain, Merriweather M.Y. *et al.* "The use of behavioural test batteries: effects of training history" *Physiol and Behav.*, 73:705-17, 2001).

8. The Wisconsin General Test Apparatus has been consistently used world wide for testing cognitive function in primates at least since the 1940s, across a range of species including Rhesus, Cynomolgus, and Squirrel monkeys, and marmosets, as demonstrated by the Harlow publication (Harlow, H.F., "A test apparatus for monkeys", *Psychological Record*, 2, 434-436, 1938), and

subsequent publications (Harlow, H.F., "The formation of learning sets", *Psychol. Rev.*, 56:51-65, 1949; Ridley, R.M. *et al.*, "A new approach to the role of noradrenaline in learning: problem-solving in the marmoset after alpha-noradrenergic receptor blockade", June, 14(6):849-855, 1981; Ridley R.M. *et al.* "Cholinergic learning deficits in the marmosets produced by scopolamine and ICV hemicholinium" *Psychopharmacology* 83, 340-345, 1984; Roberts, A.C. "Comparison of cognitive function in human and non-human primates" *Cognitive Brain Res.* 3, 319-327, 1996; and Ridley R.M., and Baker HF "Evidence for specific information processing in monkeys with lesions of the septohippocampal system" *Cortex* 33, 167-76, 1997).

9. A human pluripotent clonal cell line (designated "CX") has now been developed as well. As described in Exhibit D, which is attached hereto, these cells were conditionally immortalized using the same techniques as described in the patent application. Following confirmation of their clonality, the human conditionally immortal, pluripotent cells were implanted into the brains of rats having unilateral basal forebrain excitotoxic lesions, which mimics some of the cell loss that occurs in Alzheimer's disease and other neurodegenerative conditions. As described in Exhibit D, effects of the human cell line were assessed in comparison with several murine cell lines including the MHP36 cell line as a positive control, and with sham-grafted lesioned and non-lesioned controls. As expected, rats grafted with the murine MHP36 cell line performed significantly better than lesioned animals. However, rats receiving the human cell line showed as rapid spatial learning as controls, and were superior both to the lesion and the murine grafted groups relative to lesion-only animals in a memory task.

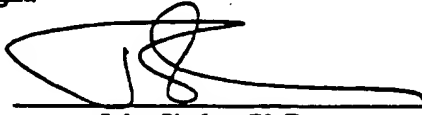
10. The Reviewer cites the Netto *et al.* publication as describing the method of our invention. However, as described above, the Netto *et al.* publication, describes the transplantation of CA1 cells from late fetal stages, e.g., embryonic day (E) 19-20; these cells are typically mature, differentiated cells and hence not pluripotent or nestin-positive. Furthermore, the cells have not been genetically modified to confer conditional immortality.

11. Based on the ability of conditionally immortal pluripotent, nestin-positive neuroepithelial cells to migrate to, and adopt the phenotype of, any damaged part of the brain, and based on the experimental data showing restored function in a variety of brain damage models, there is no reason to doubt that the invention will be applicable in treating a variety of conditions, including those characterized by cognitive deficits. Furthermore, based on the teaching of the specification and further supported by the experimental data, there is no reason to doubt that restoration of cognitive function can be achieved in a variety of mammals, including humans.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed:

  
\_\_\_\_\_  
John Sinden, Ph.D.

Date:

27th September 2002

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## Exhibit A



## CURRICULUM VITAE

**John David Sinden**

### UNIVERSITY

B.A. (Hons) in Psychology Class II Div I, The University of Sydney, 1972.

M.A. (Hons) in Psychology Class I, The University of Sydney, 1980.

Dr 3rd Cyc Neurosciences, Université Pierre et Marie Curie, Paris VI, 1983.

### SCHOLARSHIPS, FELLOWSHIPS, AWARDS

Commonwealth University Scholarship, 1969-1972.

University Medal in Psychology, The University of Sydney, 1981.

French Government Postgraduate Scholarship, 1980-1983.

European Training Programme in Brain and Behaviour short-term fellowship, 1983.

### EMPLOYMENT

A.R.G.C. Research Assistant in Psychophysiology, Macquarie University, 1973-1975.

Tutor in Psychology (full-time), The University of Sydney, 1977-1980.

Research Student, Laboratoire de Neurophysiologie Sensorielle et Comportementale, Collège de France, Paris, 1980-1983.

Post-doctoral research assistant, Department of Experimental Psychology, University of Oxford, 1983-1984.

Research worker (UFC funded), Department of Psychology, The Institute of Psychiatry, 1984-1987.

Lecturer B in Psychology (HEFC funded), Department of Psychology, The Institute of Psychiatry, 1987-1991, Confirmed 1990, Recognised Teacher of London University.

Senior Lecturer in Psychology (HEFC funded), Department of Psychology, The Institute of Psychiatry, 1991-1996.

Reader in Neurobiology of Behaviour (HEFC funded), Kings College London (Institute of Psychiatry), 1996-1998

Co-founder and Consultant, ReNeuron Limited, 1997-1998

Research Director and Board Member, ReNeuron Limited, 1998-

**John D. Sinden**

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## Exhibit B





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(57) **ABSTRACT**

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The present invention relates to the treatment of brain damage by cellular transplantation. According to one aspect of the invention, a method for treating a motor, sensory and/or cognitive deficit comprises administering a composition comprising pluripotent cells into the damaged brain in a region contra-lateral to that containing the site of damage. The cells are preferably conditionally immortal.

## TREATMENT OF BRAIN DAMAGE

### FIELD OF THE INVENTION

[0001] This invention relates to the treatment of disorders associated with damage to the brain. In particular, this invention relates to treatment of disorders by cellular transplantation into a damaged brain.

### BACKGROUND OF THE INVENTION

[0002] Stroke is the largest cause of adult disability worldwide. The incidence of stroke is about 1.3% of the US population, and 39.4% of victims show significant residual impairments, ranging from hemiplegia to restricted limb use and speech defects. Approximately 60% of strokes are caused by occlusion of the middle cerebral artery (MCAo), resulting in damage in the striatum and cortex with consequent deficits to sensory and motor systems. There is therefore a substantial clinical need for treatments that reduce or alleviate the deficits.

[0003] Typical therapies for stroke are aimed at interrupting the cascade of events that lead to intraneuronal calcium accumulation and cell death, and to provide stimulation through rehabilitation, e.g. physiotherapy, to promote intracerebral reorganisation. However, pharmacological treatments must be administered quickly to protect against cell death that typically occurs within three hours of occlusion. In addition, the therapy based on rehabilitation appears to be limited to a period of 3-6 months after stroke, after which residual disabilities do not undergo appreciable reduction.

[0004] There has been much interest recently in the possibility of transplanting new cells into the damaged neuronal system to promote repair and alleviate the disorders. One difficulty associated with cell transplantation is the need to provide clonal cell lines from different regions of the brain. This has proved to be a major difficulty in preparing cells for transplantation. WO-A-97/10329 describes the use of conditionally immortalised pluripotent neuroepithelial cells in the transplantation into the damaged brain. The neuroepithelial cells express a temperature-sensitive oncogene so that they are capable of unlimited expansion under permissive low temperatures in vitro, but cease dividing to develop into mature neural cells on implantation into the higher temperature of the brain (38° C). A particular advantage of these cells has been shown to be their ability to develop into site-appropriate neurons or glia, under the control of signals from the host brain, so that problems associated with choosing the correct tissue for transplantation is avoided. It has also been shown that the cells can migrate to the site of damage when transplanted into a region proximal to the damaged site. Therefore, the use of these cells offers a viable alternative to pharmacological treatments for repair of brain damage.

[0005] However, although the cells were shown to migrate to discrete areas of damage, focal ischaemia results in extensive damage and it is by no means certain that areas of infarction would provide a sufficiently well vascularised matrix to support the survival of grafted cells.

[0006] There is therefore the need for improvements in transplantation in order to provide cells that successfully graft into the adult damaged brain and compensate for the deficits.

### SUMMARY OF THE INVENTION

[0007] It has now been realised that pluripotent cells can successfully repair damage when administered into the side of the brain contra-lateral to that containing the site of damage.

[0008] Therefore, according to one aspect of the invention there is a method for treating brain damage comprising administering a composition comprising pluripotent cells into the damaged brain, wherein administration is into the brain hemisphere contra-lateral to that containing the site of damage.

[0009] Preferably, the pluripotent cells are neuroepithelial stem cells, in particular, those from the MHP36 clonal cell line, defined herein.

[0010] The cells are preferably conditionally immortal. Immortalisation may be achieved by the transduction of a temperature-sensitive oncogene into the cells as disclosed in WO-A-97/10329.

[0011] The advantage of administering the cells contra-laterally is that the intact (contra-lateral) region may provide a more tolerant environment for cell grafts, avoiding the inflammatory response at the site of damage which might cause cell rejection.

### DESCRIPTION OF THE INVENTION

[0012] The cells of the present invention are capable of correcting a sensory, motor and/or cognitive deficit when implanted into the brain hemisphere contra-lateral to that of the damaged part of the human brain. The term "damage" used herein includes reduction or loss of function caused by cell loss. Damage may be caused by any of a variety of means including physical trauma, hypoxia (lack of oxygen), chemical agents, for example, damage may be caused by drug abuse, and disease. The following diseases and pathological conditions are examples of diseases or conditions which result in deficits which may be treated in accordance with the present invention: traumatic brain injury, stroke, perinatal ischaemia, including cerebral palsy, Alzheimer's, Pick's and related dementing neurodegenerative diseases, multiple sclerosis, multi-infarct dementia, Parkinson's and Parkinson's-type diseases, Huntington's disease, Korsakoff's disease and Creutzfeld-Jacob disease. Amnesia, particularly following transitory global ischaemia such as after cardiac arrest or coronary bypass surgery, may also be treated in accordance with the present invention.

[0013] The present invention is particularly suited to the treatment of stroke where damage occurs primarily in one brain hemisphere e.g. due to an occlusion in the middle cerebral artery.

[0014] By "contra-lateral" it is intended that this refers to the hemisphere of the brain that does not contain the site of damage. Therefore, if there is an occlusion in the left hemisphere, then, obviously, the contra-lateral region is the right, undamaged, hemisphere.

[0015] Of course, in some instances damage may occur in both hemispheres, and in these cases the contra-lateral region is understood to be the hemisphere which exhibits least damage.

[0016] The term "pluripotent" is used herein to denote an undifferentiated cell that has the potential to differentiate

into different types or different phenotypes of cell, in particular into cells having the appropriate phenotype for the intended use. The cell type or phenotype into which such a pluripotent cell finally differentiates is at least partly dependent on the conditions in which the cell exists or finds itself.

[0017] For use in the present invention the cells should be capable of differentiating into cells appropriate to repair or compensate for damage or disease in the target area of the brain. It will be appreciated that cells for transplantation need not be capable of differentiating into all types or phenotypes of neural cells. The cells may, for example, be bipotent. However, a high degree of potency is generally preferred as this gives greater flexibility and potential for transplantation into different areas of the brain.

[0018] Suitable pluripotent cells include those known in the art as "stem cells" and those called or known as "precursor cells". In particular, neuroepithelial stem cells are suitable for use in the present invention. However, other cells may also be used. Alternative cells may be those defined as haematopoietic stem cells which may be capable of differentiating into neural cells.

[0019] The pluripotent neuroepithelial cells are advantageous, and will generally be, conditionally immortal and may be prepared as disclosed in WO-A-97/10329.

[0020] The treatment may be carried out on any mammal but the present invention is especially concerned with the treatment of humans, especially treatment with human cells, and with human cells and cell lines.

[0021] To treat a patient it is necessary to establish where damage has occurred in the brain. This may be carried out by any method known in the art, e.g. magnetic resonance imaging (MRI). Once the existence of damage has been established, whether it be in one isolated area or in several areas, treatment by implantation of cells into the contra-lateral region to that of the damaged area may be carried out, again by conventional means. The pluripotent cells may be transplanted at a single site, or preferably at multiple sites, and may be able to migrate to the site(s) of damage and, once there, differentiate in response to the local microenvironment, into the necessary phenotype or phenotypes to improve or restore function.

[0022] In addition to administering the cells into the contra-lateral region, it may also be desirable to co-administer the cells into the damaged hemisphere (ipsi-lateral region). Treatment in this manner may promote the improvement or restoration of brain function by different mechanisms.

[0023] Without wishing to be bound by theory, it may be that repair following transplantation into the contra-lateral region results from migration of the pluripotent cells into the area of damage, with the reconstitution of local circuits to restore or sustain function. It may also be that the transplanted cells augment spontaneous processes within the intact (contra-lateral) side which attempt to compensate for the damage. If the latter is correct, then it may be unnecessary for the transplanted cells to cross to the side of damage to exert an effect.

[0024] It may be possible to promote repair by encouraging the activity of particular regions of the brain. By using passive or active exercise of certain regions, it may be

possible to augment the spontaneous processes occurring after transplantation. For example, it should be possible to stimulate particular brain regions by requiring certain tasks to be performed. In doing so, the brain region may generate biological signals that aid repair.

[0025] The stimulation of the brain may be visualised using detection techniques such as magnetic resonance imaging (MRI). These techniques can be adapted to permit the patient to visualise the active brain regions, so that, through the process of biofeedback, the patient can stimulate particular regions that may encourage repair.

[0026] Preferably, treatment will substantially correct a motor, sensory and/or cognitive deficit. However, that may not always be possible. Treatment according to the present invention and with the cells, medicaments and pharmaceutical preparations of the invention, may lead to improvement in function without complete correction. Such improvement will nevertheless be worthwhile and of value.

[0027] The number of cells to be used will vary depending on the nature and extent of the damaged tissue. Typically, the number of cells used in transplantation will be in the range of about one hundred thousand to several million. Treatment need not be restricted to a single transplant. Additional transplants may be carried out to further improve function.

[0028] Methods for transplantation of cells into humans and animals are known to those in the art and are described in the literature in the art. The term "transplantation" used herein includes the transplantation of cells which have been grown in vitro, and may have been genetically modified, as well as the transplantation of material extracted from another organism. Cells may be transplanted by implantation by means of microsyringe infusion of a known quantity of cells in the target area where they would normally disperse around the injection site. Suitable excipients and diluents will be apparent to the skilled person, based on formulations used in conventional cell transplantation.

[0029] The following non-limiting example illustrates the invention.

#### EXAMPLE

[0030] Conditionally immortal pluripotent neuroepithelial cells from the MHP 36 clonal cell line were prepared as disclosed in WO-A-97/10329.

[0031] 21 Wistar rats were subjected to left intraluminal occlusion of the middle cerebral artery (MCAo-IL) under halothane anaesthesia as disclosed in Ginsberg et al, Cerebrovascular disease, 1998; Volume 1:14-35.

[0032] Following exposure of the left internal carotid artery, a 3.0 mm proline filament coated at the tip with silicon was inserted 18-20 mm up to the junction of the circle of Willis and tied in place for 60 min. Anaesthetic was discontinued after insertion of the filament, and the rat tested for neurologic deficit (contra-lateral paw flexion and circling) to establish the presence of ischaemia. Anaesthesia was resumed after 60 min for retraction of the filament to the external carotid stump, where it was left in place, the excess filament trimmed off, and the wound sutured. Neurologic and health status were monitored for a week, until normal feeding was seen and post-operative weight regained. Control rats (n=11) were sham operated by exposure of the left internal carotid artery only.

## Exhibit C

# Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field

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## Summary

Common marmosets (*Callithrix jacchus*,  $n = 18$ ) were trained to discriminate between rewarded and non-rewarded objects (simple discriminations, SDs) and to make conditional discriminations (CDs) when presented sequentially with two different pairs of identical objects signifying reward either in the right or left food well of the Wisconsin General Test Apparatus. After bilateral *N*-methyl-D-aspartate (0.12 M) lesions through the cornu ammonis-1 (CA1) field (7  $\mu$ l in five sites), marmosets showed profound impairment in recall of CDs but not SDs, and were assigned to lesion only, lesion plus CA1 grafts and lesion plus Maudsley hippocampal cell line, clone 36 (MHP36) grafts groups matched for lesion-induced impairment. Cell suspension grafts (4  $\mu$ l, 15–25 000 cells/ $\mu$ l) of cells dissected from the CA1 region of foetal brain at embryonic day 94–96, or of conditionally immortalized MHP36 cells, derived from the H-2K<sup>b</sup>-tsA58 transgenic mouse neuroepithelium and labelled with [<sup>3</sup>H]thymidine, were infused at the lesion sites. The lesion plus MHP36 grafts group was injected five times per week with cyclosporin A (10 mg/kg) throughout testing. Lesion, grafted and intact control marmosets ( $n = 4$ –5/group) were tested on recall of SDs and CDs learned before lesioning and on acquisition of four new CDs over a 6-month period. Lesioned animals were highly

impaired in recall and acquisition of CD tasks, but recall of SDs was not significantly disrupted. Both grafted groups of marmosets showed improvement to control level in recall of CDs. They were significantly slower in learning the first new CD task, but mastered the remaining tasks as efficiently as controls and were substantially superior to the lesion-only group. Visualized by Nissl staining, foetal grafts formed clumps of pyramidal-like cells within the denervated CA1 field, or jutted into the lateral ventricles. MHP36 cells, identified by  $\beta$ -galactosidase staining and autoradiography, showed neuronal and astrocytic morphology, and were distributed evenly throughout the CA1 region. The results indicate that MHP36 cell grafts are as functionally effective as foetal grafts and appear to integrate into the host brain in a structurally appropriate manner, showing the capacity to differentiate into both mature neurons and glia, and to develop morphologies appropriate to the site of migration. These findings, which parallel the facilitative effects of foetal and MHP36 grafts in rats with ischaemic CA1 damage, offer encouragement for the development of conditionally immortal neuroepithelial stem cell lines for grafting in conditions of severe amnesia and hippocampal damage following recovery from cardiac arrest or other global ischaemic episodes.

**Keywords:** marmoset; hippocampal lesions; conditional discriminations; intracerebral transplants; neuroepithelial stem cells

**Abbreviations:** CA = cornu ammonis; CD = conditional discrimination; MHP36 = Maudsley hippocampal cell line, clone 36; NMDA = *N*-methyl-D-aspartate; SD = simple discrimination

## Introduction

The hippocampus, particularly the hilar and cornu ammonis-1 (CA1) fields, is highly susceptible to ischaemic brain injury (Schmidt-Kastner and Freund, 1991) following interruption of cerebral blood flow. Clinical studies and experimental lesions in animals have demonstrated that damage to the hippocampus is associated with deficits in learning and memory. For example, up to 40% of patients recovering from out-of-hospital heart attack have shown moderate to severe memory loss (Roine *et al.*, 1993; Grubb *et al.*, 1996), whilst both neuroimaging and post-mortem studies have revealed selective hippocampal damage, particularly in the CA1 field, in patients with memory impairments following ischaemic episodes (Zola-Morgan *et al.*, 1986; Squire *et al.*, 1990; Kartsounis *et al.*, 1995; Rempel-Clower *et al.*, 1996). Impairments in learning new information (anterograde amnesia) have been most prominent, but recent findings have shown that temporally graded retrograde amnesia may also be evident (Kartsounis *et al.*, 1995; Rempel-Clower *et al.*, 1996).

Global ischaemia is modelled in a controlled and reproducible way by four vessel occlusion in rats in which the vertebral arteries are coagulated and the carotids transiently occluded for periods of 5–30 min (Pulsinelli *et al.*, 1982). Global ischaemia exhibits duration-related trends both in the extent of cell loss and cognitive impairment (Pulsinelli *et al.*, 1982; Nunn and Hodges, 1994; Nelson *et al.*, 1997a, b), shown chiefly by robust spatial learning and spatial working memory deficits. Although results from correlational studies have been variable (Nunn and Hodges, 1994), recent findings demonstrate that the extent of hippocampal CA1 cell loss induced by four vessel occlusion in rats is related to the extent of the behavioural deficit and confirm that this field is crucially involved in ischaemic learning and memory impairment (Olsen *et al.*, 1994; Nelson *et al.*, 1997a, b; Block and Schwarz, 1998).

Primate models of global ischaemia, such as constriction of ascending arteries using a neck cuff, combined with hypotension (Zola-Morgan *et al.*, 1992) are more variable in their effects than four vessel occlusion in rats and neurological deficits are seen at durations which produce minimal cognitive impairments (Scheller *et al.*, 1992). Nevertheless, this method has been shown to produce damage to CA1 and CA2 fields and deficits in 'amnesia sensitive' recognition memory tasks, such as delayed non-matching to sample which is as great as that seen in monkeys with hippocampal and parahippocampal cortex lesions. Surgical occlusion of the posterior cerebral artery, which supplies the posterior hippocampus, has also been shown to impair delayed non-matching to sample performance in rhesus monkeys that sustained ischaemic CA1 and CA2 damage (Bachevalier and Mishkin, 1989), although the infarct size was highly variable. Ridley and colleagues (Ridley *et al.*, 1995) demonstrated that *N*-methyl-D-aspartate (NMDA) infused along an angled trajectory resulted in discrete and reproducible lesions of the CA1 field in marmosets, thus providing an alternative method to model

effects of ischaemic CA1 cell loss. Cognitive deficits arising from this lesion resembled those shown by animals with damage to septal/diagonal band and fimbria–fornix inputs to the hippocampus, but not by lesions of the entorhinal cortex (Ridley *et al.*, 1995, 1996). These deficits were prominent in visuospatial conditional discrimination tasks (CDs) when different pairs of identical objects determined whether the reward was to be found in the left or right food well. Relatively little impairment was seen in serial spatial reversal, concurrent discrimination or pattern discrimination tasks despite their demands on memory. Ridley and Baker suggest that damage to the hippocampus and fornix impair encoding of information into long-term memory in situations which require simultaneous attention to multiple features of stimuli as well as comparisons with previous stimuli (Ridley and Baker, 1997).

Treatments to alleviate effects of global ischaemic brain damage have typically focused on agents that might interrupt the cascade of events leading to intraneuronal calcium accumulation, the major factor precipitating cell death (Meldrum, 1990), including antagonists at different subtypes of glutamate receptor, calcium and sodium channel blockers, calcium chelators and free radical scavengers (Hunter *et al.*, 1995). Apart from a few exceptions (Block and Schwarz, 1998), significant protection against CA1 cell loss with durations of occlusion above the threshold for cognitive deficit (~10 min with four vessel occlusion in rats) has been difficult to demonstrate. Treatments must usually be given shortly after occlusion and may rescue cells at risk, but they cannot reverse the effects of cell death, which limits their therapeutic potential (Hunter *et al.*, 1995). Use of cerebral transplants to alleviate memory deficits induced by hippocampal damage offers the advantage that the aim of transplanting is to promote functional recovery from damage that has already occurred. Several lines of evidence suggest that foetal hippocampal grafts achieve considerable functional integration into the host brain. Field and colleagues demonstrated that foetal grafts within the hippocampus are contacted by host neurons in an appropriate laminar fashion, provided that cells are homotypically replaced within the lesion site, suggesting that foetal grafts may restore information flow around the hippocampus in a relatively point-to-point manner (Field *et al.*, 1991). This suggestion has been supported by findings that grafts of dentate granule cells, but not CA1 cells, restore long-term potentiation (a correlate of learning) in rats with dentate gyrus lesions (Dawe *et al.*, 1993). In terms of cognitive effects, foetal grafts have shown remarkable specificity. CA1, but not CA3 or dentate granule grafts, improved the performance of rats (subjected to four vessel occlusion) that sustained marked ischaemic CA1 cell loss and showed deficits in a variety of spatial learning and working memory tasks in both a water maze and three-door runway (Netto *et al.*, 1993; Hodges *et al.*, 1996, 1997). The recent findings in marmosets that grafts of

foetal CA1 cells are highly effective in improving recall of conditional tasks learned before lesioning, and also modestly improve CD task learning in animals with profound deficits after CA1 lesions, indicate that cognitive improvement after foetal hippocampal grafts extends to primate species (Ridley *et al.*, 1997). Thus, in terms of eventual clinical application to patients suffering memory loss after ischaemic or traumatic hippocampal damage, where current cerebroprotective drug treatments offer limited therapy, grafting strategies have several advantages: grafts can be accurately targeted to a discrete region of brain damage, their growth can be visualized by neuroimaging techniques with concurrent neuropsychological assessments and, unlike neurodegenerative disease conditions, grafts will not be subjected to the effects of progressive degeneration of the host environment.

Grafts of foetal cells are not likely to provide a viable therapeutic approach for ethical and practical reasons, although they have been important as 'proof of concept' in animal models (Sinden *et al.*, 1995). Several alternative strategies are under development, including genetically engineered cells to deliver neurotrophins or enzymes to specified brain regions, encapsulated cells and cultured stem cells with pluripotent developmental capacity (Gage *et al.*, 1995; McKay, 1997). Three requirements are critical for development of non-foetal donor stem cells. These are (i) the capacity to proliferate in culture but to cease dividing and develop into mature neurons or glia after transplantation into the brain (conditional immortality); (ii) responsiveness to inductive signalling which enables grafted stem cells to differentiate according to the region into which they are implanted (multipotency); and (iii) clonal derivation providing cells of a known lineage in which it is possible to understand and manipulate signals that trigger differentiation along different developmental routes. Recently, Sinden and colleagues have shown that the FGF-2 (fibroblast growth factor-2) responsive Maudsley hippocampal cell line, clone 36 (MHP36), cloned from the E 14 H-2K<sup>b</sup>-tsA58 transgenic mouse (Jat *et al.*, 1991) hippocampal neuroepithelium, possesses these characteristics (Sinden *et al.*, 1997). This cell line is conditionally immortalized since the 'immortomouse' expresses the temperature sensitive large T antigen gene in every cell, so that cells derived from this mouse divide at a low (33°C) temperature *in vitro*, but differentiate into mature cells at brain temperature (37–39°C) on implantation. MHP36 cells are multipotent and have been shown to develop into both neurons and glia *in vitro* and *in vivo* (Kershaw *et al.*, 1994; Sinden *et al.*, 1997).

Hippocampal damage following four vessel occlusion or excitotoxic lesions would be expected to provide a favourable environment for grafted MHP36 cells since cell death has been shown to trigger time-dependent changes in the expression of trophic factors, cytokines, neurotrophins and their high affinity receptors (Takeda *et al.*, 1993; Endoh *et al.*, 1994; Maeda *et al.*, 1994) which normally assist in remodelling of circuits following ischaemic or traumatic injury (Lindvall *et al.*, 1994). These factors would be expected to promote

site-specific differentiation of grafted cells (Whittemore and White, 1993). Time-course studies of MHP36 grafts in four vessel occlusion ischaemic rats (Sinden *et al.*, 1997) showed that grafted cells labelled with antibodies to  $\beta$ -galactosidase migrated to repopulate the area of CA1 cell loss within 4 weeks of implantation and adopted both neuronal pyramidal cell-like and astrocytic morphologies. This alignment of MHP36 cells within the area of host CA1 cell loss is in marked contrast to foetal cells, which typically mass around the injection site and migrate in clumps along the corpus callosum in rats, rather than seeking the CA1 field. Moreover, ischaemic rats with MHP36 grafts showed substantial recovery of spatial learning and working memory towards control level and performed as well as those with CA1 foetal grafts (Sinden *et al.*, 1997), suggesting a comparable capacity for functional reconstruction of the hippocampus.

The efficacy of MHP36 grafts in rats with CA1 damage prompted us to assess their functional effects in marmosets with CA1 lesions, which had proved responsive to ameliorative effects of foetal grafts on CD task learning. The present experiment therefore aimed to replicate and extend the findings of Ridley and colleagues (Ridley *et al.*, 1997) by comparing lesioned marmosets that had CA1 foetal grafts or MHP36 grafts with lesion-only and intact controls. We aimed to test the recall of simple discriminations (SDs) and CDs after lesioning to establish the lesion deficit and to assign animals of comparable impairment to the three lesion groups. Following transplantation we aimed to assess both long-term recall and the learning of several new discrimination tasks to probe the stability of both lesion-induced deficits and of any graft-induced recovery.

## Material and methods

These experiments were carried out in accordance with the UK Scientific Procedures Act, 1986, and guidance notes for good practice issued by the UK Home Office and by the Animal Welfare and Research Advisory Committee of the Institute of Psychiatry.

## Animals

Eighteen laboratory-born common marmosets (*Callithrix jacchus*), 10 males and eight females aged between 2 and 5 years and weighing 350–475 g, were housed one to three per cage in a light (lights on 07.30–19.30) and temperature (22–25°C) controlled vivarium. They were fed with mixed fresh fruit and marmoset jelly once a day after training, and marmoset chow was available *ad libitum*. Three times a week they were given pellets made of Cytacyn syrup [vitamin B<sub>12</sub>, Goldshield Pharmaceuticals (Europe) Ltd, Croydon, Surrey, UK] and vitamin D<sub>3</sub> oil (Vetoquinol UK Ltd, Bicester, Oxon, UK) mixed with baby cereal to form a soft dough. This mixture was avidly consumed and small pieces were also used as rewards for most of the animals throughout training.

### Behavioural training and testing

Marmosets were trained in the Wisconsin General Test Apparatus to discriminate between objects mounted on disks placed above two food wells. The animal entered a transport box placed in the home cage which was put in front of the screen door of the apparatus. At the start of a trial the screen was raised to reveal the two objects; the monkey reached through the grill of the transport box to touch one of the objects. Displacement of the 'correct' object revealed food underneath, displacement of the 'incorrect' object yielded no reward; the screen was lowered and the objects replaced for the next trial. Typically, animals completed 30–40 trials in a session of 30 min. Animals were trained in two types of task: SDs where one of two objects was always rewarded, and visuospatial CDs using two different pairs of identical objects presented in a pseudorandom order. With one pair the reward was always on the left, with the other pair on the right. All animals were initially trained to a criterion of 90 out of 100 correct trials on SD. This 'shaped' the animals to perform in the apparatus and established a stable performance. The animals were then tested on two examples of the SD task (using new objects for each example) and two examples of the CD task to a criterion of 27 out of 30 correct choices. SD tasks were mastered in ~50 trials and CD tasks in 100–150 trials. Animals were matched for learning ability and assigned to control ( $n = 5$ ) or lesion ( $n = 13$ ) groups. After surgery, all animals were tested on retention of one of the SD tasks and one of the CD tasks in order to establish the lesion-induced impairment. In order to qualify for subsequent behavioural analysis, lesioned animals had to have an SD score at or below, and a CD score above their acquisition scores for these two tasks. This would indicate that they did not have a large amount of collateral damage, but they probably did have a substantial bilateral CA1 lesion. Two lesioned animals, additional to the 13 that completed the experiment, were excluded because they were not impaired on the CD task. The remaining lesioned animals were matched for lesion induced learning impairment and allocated to three groups: lesion only ( $n = 5$ ), lesion plus CA1 grafts ( $n = 4$ ), lesion plus MHP36 grafts ( $n = 4$ ). Following grafting, animals were tested for retention of another SD and CD task learned before lesioning. They were then tested with four new CD tasks over a 6-month period. Animals which did not achieve criterion in 300 trials were deemed to have 'failed' and moved to the next task.

### Surgery

Animals were operated in batches of 2–3 so that groups of lesion and lesion plus grafts were tested simultaneously. Control marmosets were not operated. For lesioning, marmosets were pre-treated with dexamethasone [2.0 mg/kg, i.m. (intramuscular); Merck, Sharp and Dohme Ltd, Harlow, UK] to reduce oedema, and anaesthetized with alphaxalone-alphadolone (Saffan, 18 mg/kg, i.m.; Schering-Plough Ltd,

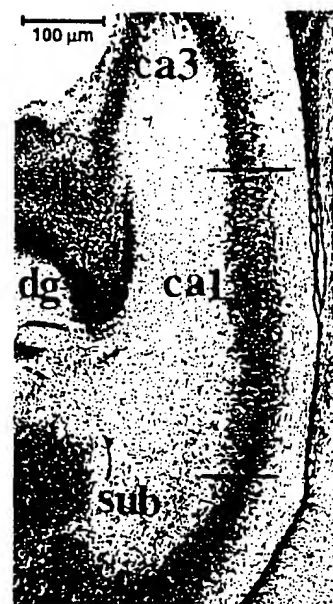


Fig. 1 Nissl stained coronal section through foetal (embryonic day 94–96) hippocampus showing approximate lines of dissection of the CA1 field. At this posterior level and stage of development the pyramidal cell layer is narrow and densely packed relative to the adult CA1 field (cf. Fig. 5A). dg = dentate gyrus; sub = subiculum; scale bar = 100 μm.

UK). Stereotaxic co-ordinates were set up to allow the angled penetration of a 10 μl Hamilton syringe through the CA1 field via a burr hole drilled in the occiput, as described by Ridley and colleagues (Ridley *et al.*, 1995). Five equidistant infusions of 0.7 μl of NMDA (0.12 M in 0.9% saline, pH adjusted to 7.4 with 1 N NaOH) were made through this trajectory. Following surgery animals were monitored for body temperature for several hours until conscious and feeding or drinking had been observed. Marmosets remained in an incubator overnight, with food and water available. They were then housed singly in post-operative cages until normal feeding and activity were apparent, and then returned to their home cages. About a week after surgery lesioned animals were tested with one SD and one CD task learned pre-operatively. Animals assigned to transplant groups then received cell suspension grafts along the same sites as the lesion, following the same procedure, ~3 weeks after lesioning.

For foetal grafts, twin or triplet embryos (3.5–4 cm crown–rump length) were removed at embryonic day 94–96 (calculated as 106–108 days after previous parturition) by laparotomy and the mother was stitched and placed in an incubator for recovery. Foetal heads were removed, and placed in tubes containing 1 mM *N*-acetyl-L-cysteine (Sigma, Poole, Dorset, UK) in Hanks' balanced salt solution (Sigma), packed in ice and transferred to the home laboratory of the marmosets awaiting surgery (~3 h). Brains were then dissected on ice using an operating microscope. The CA1 field of the hippocampus was removed (Fig. 1) and cut into fragments, combining tissue from all available foetuses, since each recipient received all the CA1 tissue from one donor. Tissue



was incubated for 20 min at 37°C in Hanks' balanced salt solution containing 0.1% trypsin (Sigma) and 0.01% DNAase (Sigma) and 1 mM *N*-acetyl-L-cysteine. Digestion was stopped by replacing the supernatant with Hanks' balanced salt solution/*N*-acetyl-L-cysteine containing 0.01% soybean trypsin inhibitor and 0.06% bovine serum albumin, fraction V (Sigma) for 10 min at room temperature. Tissue was washed several times in the Hanks balanced salt solution/*N*-acetyl-L-cysteine medium and dissociated into a cell suspension by gentle movement through a small bore fire polished pipette. Viable cell density was counted before and after transplantation by trypan blue exclusion in a haemocytometer. The final suspension (50 µl) contained 15 000–23 000 cells/µl. Initial viability was ~95% and fell to ~80% after transplant surgery. Cells were injected at five points along the lesion trajectory bilaterally (4 µl/site) using the same procedure as for lesioning.

The derivation of MHP36 cells has been described by Sinden and colleagues (Sinden *et al.*, 1997). Cells for grafting were brought up from frozen stock (passage 36) and suspended at a concentration of 25 000 cells/µl in 1 mM *N*-acetyl-L-cysteine in Hanks' balanced salt solution. Two days before transplantation cells were pulsed with 0.5 µCi/ml [<sup>3</sup>H]thymidine to permit identification by autoradiography. This was necessary because expression of the  $\beta$ -galactosidase marker has been found to be low after ~6 weeks of survival, and therefore this label may not be optimal for long-term behavioural studies. As with foetal grafts, MHP36 cells were suspended in Hanks' balanced salt solution/*N*-acetyl-L-cysteine and injected at five sites (4 µl/site, 24–25 000 cells/µl) along the lesion trajectory. Cell viability was 98% before transplantation and 85% by the end of surgery. Grafted marmosets received i.m. injections of cyclosporin A (Sandimmun, Sandoz Ltd, Frimley, Surrey, UK), 10 mg/kg suspended in Cremophor EL (Sigma) five times a week until sacrifice.

Testing commenced 12 weeks after grafting in animals with foetal grafts, and 6 weeks after grafting in animals with MHP36 grafts, following evidence from previous time-course studies in rodents of the integration of grafted foetal (Mudrick and Baimbridge, 1991) and MHP36 (Sinden *et al.*, 1997) cells into damaged hippocampus. Lesioned and non-lesioned controls were tested along with the grafted animals. Table 1 gives the approximate time-course of the experiments, but it should be noted that animals were run in overlapping batches of two to four from different groups and therefore not during the same period of time.

### Histology

At the end of behavioural testing animals were perfused with 4% paraformaldehyde (Sigma) and the brains removed for wax embedding. Sections of 7 µm were cut through the hippocampus and serial sections taken for Nissl and glial fibrillary acidic protein staining, to identify graft masses and pyramidal or astrocyte-type cells within grafts, and host glial

**Table 1** Duration of the phases of the experiment

Weeks (approx.)	Procedures
1–8	Phase 1: acquisition of SDs and CDs
9	Lesion surgery
11–12	Phase 2: post-operative retention of SDs and CDs
13	Transplant surgery
19	Phase 3: post-transplant retention of SDs and CDs in marmosets with MHP36 grafts
21–31	Phase 4: acquisition of new CDs in marmosets with MHP36 grafts
25	Phase 3: post-transplant retention of SDs and CDs in marmosets with CA1 grafts
26–41	Phase 4: acquisition of new CDs in marmosets with CA1 grafts

Animals were run in batches of 2–4 marmosets from different groups at staggered intervals, but all animals followed this approximate time-course. The overall length of time for each phase was determined by the slowest animals in each batch.

reactivity to lesion damage and the presence of grafts. Sections from brains with MHP36 grafted cells were additionally treated for autoradiography and immuno-reactivity to  $\beta$ -galactosidase and  $\beta$ III-tubulin (monoclonal anti- $\beta$ -tubulin isotype III, Sigma) to identify labelled grafted cells and whether they expressed neuronal characteristics.

### Data analysis

The number of trials to criterion (maximum 300) in the SD and CD tasks was compared across groups by two-factor (group  $\times$  task) analyses of variance (Genstat V PC) for acquisition and each retention phase (phases 1–3). A repeated measures analysis was used for comparison across groups for the acquisition of four new CD tasks after transplantation (phase 4). Grafted groups were compared with lesioned and intact controls by Newman-Keuls comparison of means, and orthogonal trends were used to detect systematic changes in performance in phase 4.

### Results

#### Acquisition of SDs and CDs (phase 1)

There were no differences between groups in the mean number of trials required to learn SDs (~40–50) or CDs (100–150). All marmosets found SD tasks easier to learn than CD tasks [ $F(1,14) = 25.53, P < 0.001$ ] so there were no interactions between groups and task (Fig. 2, phase 1).

#### Post-lesion retention of SDs and CDs (phase 2)

All lesioned animals were substantially impaired in retention of CDs but not SDs. Lesioned groups differed from controls ( $P < 0.01$ ) for the CD but not the SD task. Marmosets assigned to the lesion-only and the two grafted groups did not differ from each other in the extent of impairment in recall of the CD task (Fig. 2, phase 2). This selective

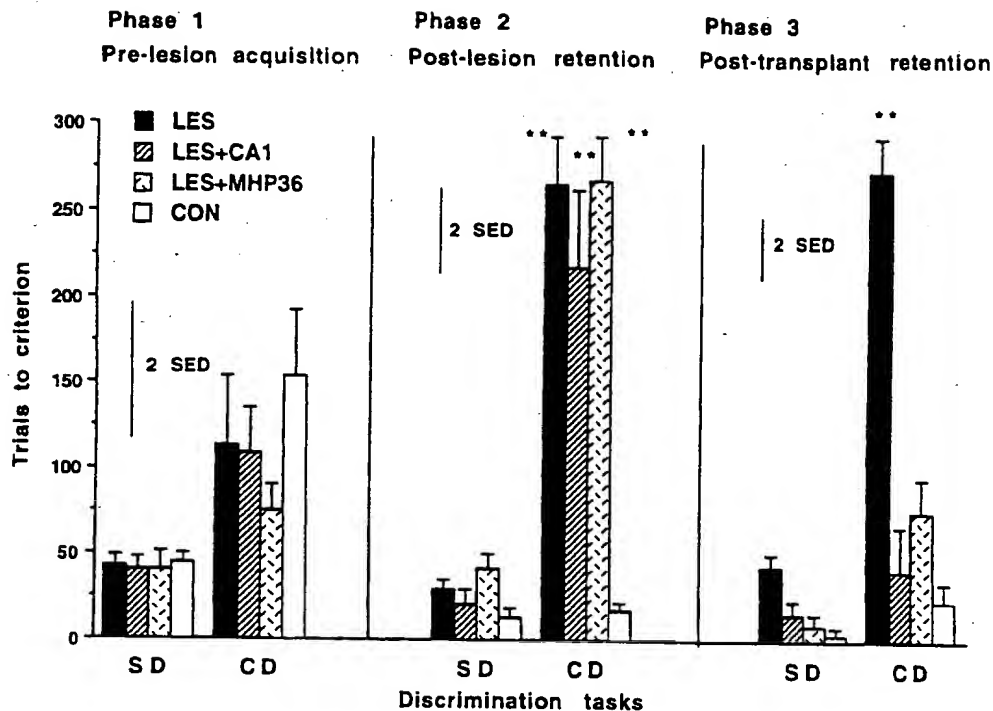


Fig. 2 Mean ( $\pm$ SEM) number of trials to criterion (27 out of 30 correct choices) in SDs and CDs in phases 1–3. Phase 1: pooled results for learning of two tasks before lesioning showing that monkeys in all groups required a similar number of learning trials (~50 for SDs, 100–150 for CDs). Phase 2: retention of one of these tasks in control and lesioned marmosets. Note the selective and substantial lesion deficit in the CD task relative to non-lesioned controls (CON). Marmosets assigned to lesion-only (LES), lesion plus CA1 foetal grafts (LES + CA1) and lesion plus MHP36 grafts (LES + MHP36) groups did not differ in the extent of lesion-induced impairment. Phase 3: post-graft retention of a second SD and CD task learned before lesioning. Lesion-only marmosets continued to show a substantial deficit in recall of the CD task but not the SD task relative to controls. Lesioned marmosets with foetal or MHP36 grafts did not differ from controls in the number of trials to criterion in the CD task despite their substantial impairment before transplantation. SED bars show twice the standard error for the difference in means between groups. Difference from controls,  $**P < 0.01$ .

lesion-induced CD deficit was shown by substantial differences between groups [ $F(3,14) = 26.08$ ,  $P < 0.001$ ] and the highly significant interaction between groups and task [ $F(3,14) = 22.22$ ,  $P < 0.001$ ]. The difference between tasks seen in acquisition was magnified by the lesion-induced CD impairment [ $F(1,14) = 179.99$ ,  $P < 0.001$ ].

#### Post-transplant retention of SDs and CDs (phase 3)

After transplantation, groups with CA1 and MHP36 grafts recalled CDs as efficiently as controls, but the lesion-only group remained substantially impaired relative to controls and both of the grafted groups ( $P < 0.01$  in all comparisons; Fig. 2, phase 3). The lesion-only group also required more trials to criterion for the SD task than controls, but this difference was not significant. As a result of the continued CD impairment in lesion-only animals, there were marked differences between groups [ $F(3,14) = 61.79$ ,  $P < 0.001$ ] and a substantial group  $\times$  task interaction [ $F(3,14) = 34.04$ ,  $P < 0.001$ ]. As in post-lesion retention, the difference between tasks was highly significant [ $F(1,14) = 103.08$ ,  $P < 0.001$ ].

#### Acquisition of CDs after transplantation (phase 4)

Learning scores (trials to criterion) of lesioned groups with and without grafts and the control group were compared in four new CD tasks over a period of 5–6 months after transplantation (Fig. 3). There were substantial differences between groups [ $F(3,14) = 30.85$ ,  $P < 0.001$ ] and a significant linear trend of improvement over tasks [ $F_{lin}(1,40) = 27.54$ ,  $P < 0.001$ ; Fig. 3]. On the first task all the lesioned groups were impaired relative to controls ( $P < 0.01$ ). However, the lesion-only group was also significantly more impaired than each of the grafted groups ( $P < 0.01$ ). Performance of the grafted groups was thus intermediate between control and lesion level at this point. On the subsequent CD acquisition tasks (CDs 2–4) both the lesion plus CA1 grafts and the lesion plus MHP36 grafts groups improved to control level, whilst the lesion-only group continued to show profound impairment. Thus, the lesion group differed substantially from the control and both of the grafted groups throughout acquisition of CDs 2–4 ( $P < 0.01$ ) and there was no evidence for a reduction in the lesion deficit during the training period.

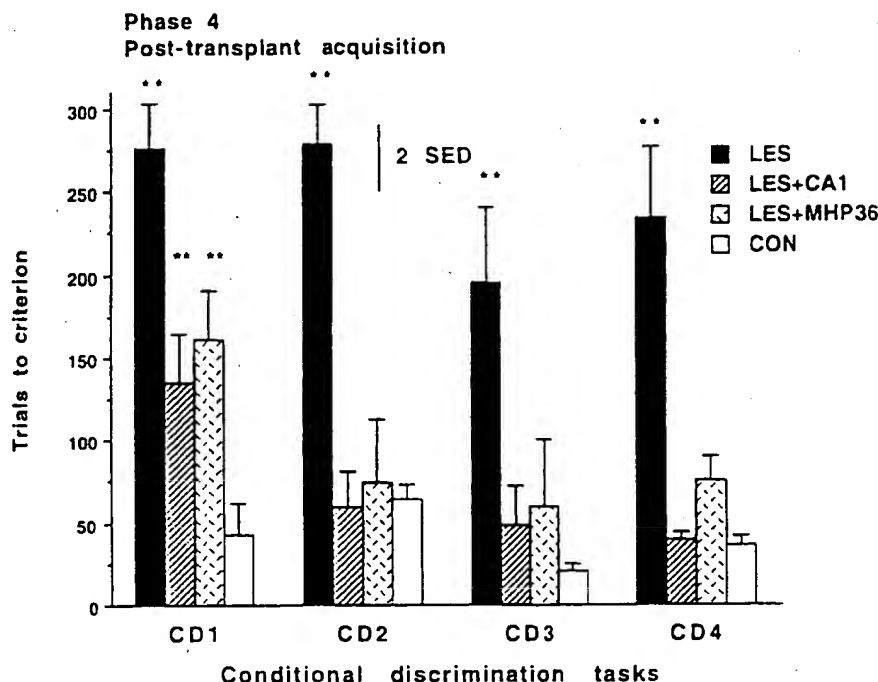


Fig. 3 Mean ( $\pm$ SEM) number of trials to criterion (27 out of 30 correct choices) during acquisition of four new CD tasks in phase 4. The lesion control group remained substantially impaired relative to control and grafted groups throughout training. Marmosets with CA1 or MHP36 grafts were also impaired relative to controls in the first CD task, although they learned significantly more rapidly than the lesioned controls. For CD tasks 2–4 neither grafted group showed a learning impairment. Groups as in Fig. 2. SED bar shows twice the standard error for the difference in means between groups over the repeated measures for tasks. Difference from controls,  $**P < 0.01$ .

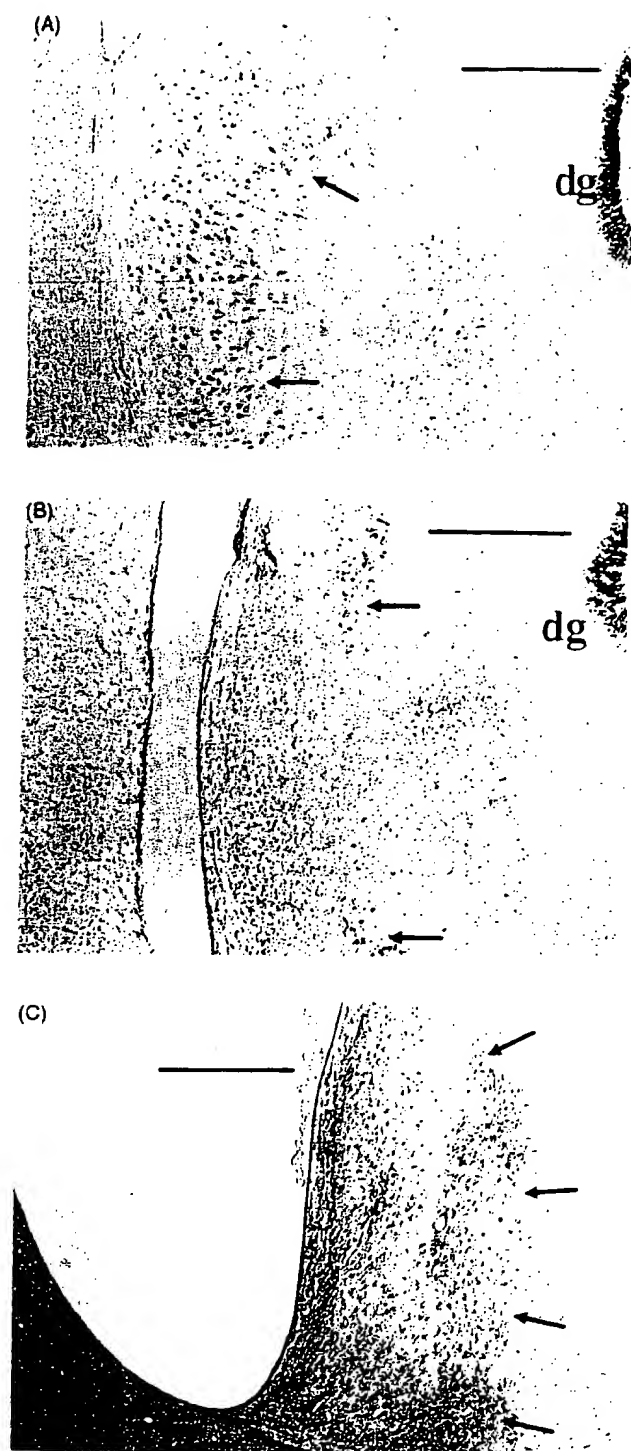
## Histological assessment

### Lesions

In all animals lesions were associated with a shrinkage of the hippocampus and marked enlargement of the lateral ventricles (Fig. 4B and C). Lesion sites were characterized by loss of CA1 pyramidal cells and marked glial reactivity (Fig. 4C), shown by dense glial fibrillary acidic protein staining within the lateral hippocampus (CA1 field) and the cortical margins of the lateral ventricles. This reactivity was comparable in lesioned and grafted animals, and therefore grafts do not appear to have modified activated glial response to neurotoxic injections.

Loss of hippocampal cells was assessed in Nissl stained coronal sections in lesioned and control animals by cell counts at level AP (anterior–posterior) 4.5 mm (Stephan *et al.*, 1980) at the centre of the lesion axis. CA1 cell counts were carried out within a field measuring  $0.62 \text{ mm}^2$ , which was placed over the CA1 area. It was possible to exclude transplanted cells from the counted cells for several reasons. Foetal cells in all but one case seeded outside the CA1 sector used for counting. In the one animal with grafted cells within this field, the graft structure was clearly delineated and grafted cells differed in organization and density from normal host CA1 cells. The grafted murine MHP36 cells in the field were much smaller than marmoset CA1 cells, and as only the large CA1 pyramidal cells were counted, small grafted cells, with nuclei only half the diameter of host CA1 cells, were excluded. Counts showed that bilateral numbers of CA1

cells, averaging  $\sim 300$  in controls, fell to means ranging from 9 (lesion plus MHP36 grafts) to 56 (lesion plus CA1 grafts) in the lesioned groups, a difference that was highly significant [ $F(3,14) = 25.45$ ,  $P < 0.0001$ ; Table 2]. Mean differences between controls and the three lesioned groups were robust ( $P < 0.001$ ), whereas none of the lesioned groups differed significantly from each other. Lesion sites at AP 4.5 mm, shown in Fig. 5, provide sections with the least and most extensive CA1 cell loss across all animals within in each group. In most lesioned animals there was extensive (80–90%) bilateral cell loss through the anterior–posterior axis of the CA1 field. In one monkey with lesion-only and two with CA1 grafts, a high proportion of cells survived on one side (i.e. 50–80% of control level at AP 4.5 mm; Fig. 5), but very few CA1 cells were detected on the other side. In monkeys with MHP36 grafts the lesions almost totally obliterated pyramidal cells throughout the CA1 field. Thus, the functional recovery in grafted animals cannot be attributed to the chance occurrence of less severe lesions than in animals with lesions alone. In addition to CA1 cell loss, four animals (two from the lesion plus CA1 grafts group and one from each of the lesion plus MHP36 grafts and lesion-only groups) showed unilateral damage to the dentate gyrus, and in three animals (two from the lesion plus MHP36 grafts group and one lesion-only animal) damage extended unilaterally into the subiculum. The two animals that were excluded on behavioural grounds (see Material and methods) were found, on histological examination, to have sustained only unilateral CA1 lesions.



**Fig. 4** Coronal sections through control and hippocampal lesioned brain: (A) Nissl stained section from a non-lesioned control showing the broad and even distribution of CA1 pyramidal cells (arrows). (B) Nissl stained section from a lesioned marmoset showing marked reduction of CA1 pyramidal cells from the CA1/CA3 junction to the start of the ventral curvature where the CA1 merges with the subiculum (arrows). (C) Section stained for reactivity to glial fibrillary acidic protein. Marked glial response (arrows) is seen clearly delineating the lesion site. This occurred in all lesioned animals whether grafted or not. All three sections were taken ~3.5–4 mm anterior to the interaural line. Note the enlargement of ventricles in lesioned animals (B and especially C) relative to the control (A). dg = dentate gyrus. Scale bar = 500  $\mu$ m.

**Table 2** Mean ( $\pm$  SEM) CA1 cell counts at AP 4.5 mm before the inter-aural line in control, lesioned and grafted marmosets

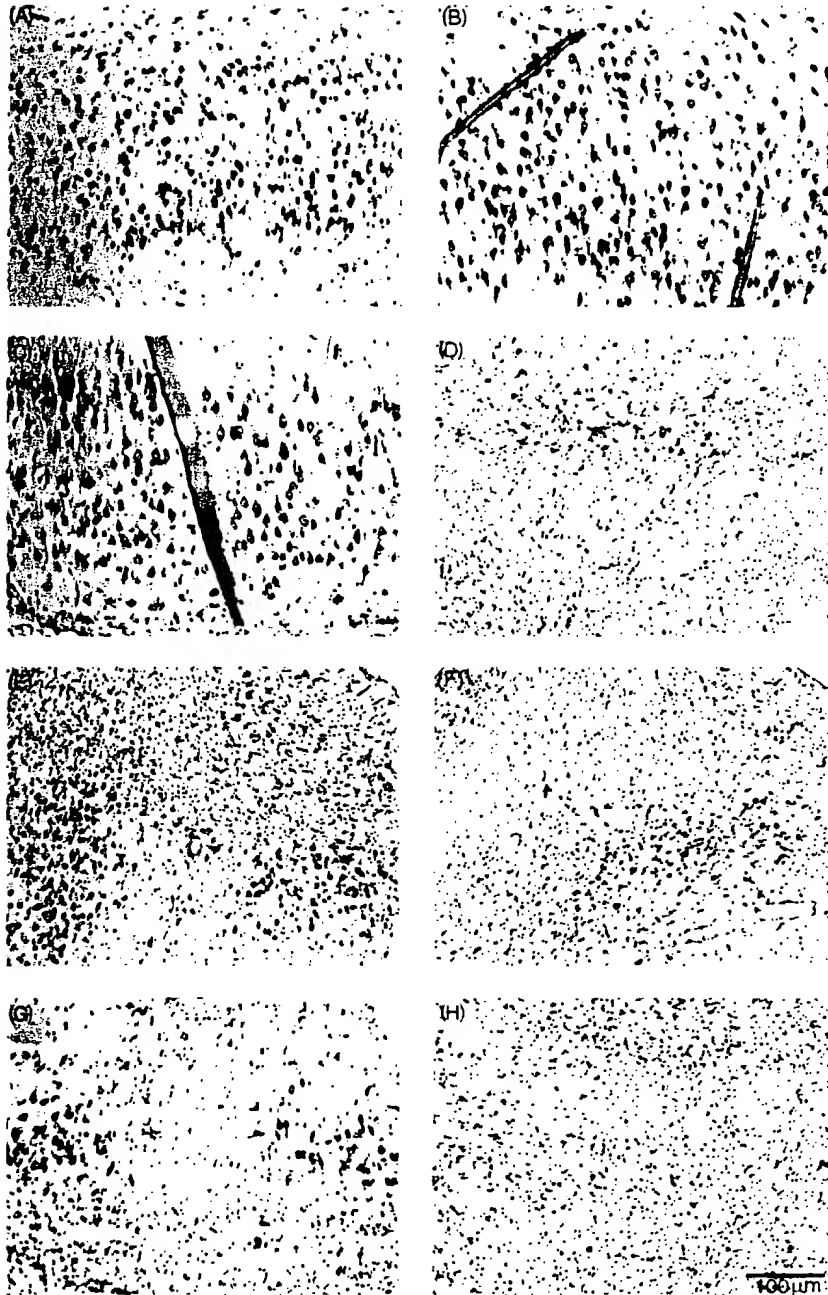
Group	Number of cells	Difference from controls ( <i>P</i> )
Control	297.0 $\pm$ 9.0	–
Lesion	23.5 $\pm$ 20.6	<0.001
Lesion plus CA1 grafts	56.5 $\pm$ 32.9	<0.001
Lesion plus MHP36 grafts	8.9 $\pm$ 7.1	<0.001

### Foetal grafts

Nissl stained sections clearly revealed grafted cells of CA1-like morphology in all four animals that received transplants, when examined ~8 months after grafting. Foetal grafts were of two types: those that formed dense swirls within the lesioned CA1 field (Fig. 6) and those that formed lobular masses attached to the edge of the CA1 field (Fig. 7), jutting into the enlarged ventricular space and, in some cases, forming an artificial bridge between the hippocampus and the white matter adjacent to the temporal cortex (Fig. 7). In one animal grafts on both sides were of the lobular type, sited at anterior levels (~4.5–6.5 mm anterior to the inter-aural line). In the second marmoset grafts were seen within central and posterior regions of the hippocampal lesion site (~1.5–4.5 mm anterior to the inter-aural line). In the third animal a ventricular graft was sited in an anterior position and a smaller intrahippocampal graft was located at a posterior site (~1.5–2.5 mm anterior to the inter-aural line). In the fourth animal the right hippocampus was damaged during processing so that, although there appeared to be grafted cells on the edge of the damaged CA1 field, these could not be clearly identified. This animal had an extensive anterior lobular graft (~4.0–6.5 mm anterior to the inter-aural line) attached to the left hippocampus. Since improvement in the recall and learning of CDs was not significantly different from controls in all animals with foetal grafts in all CD tasks except one, these grafts seemed to be adequate to produce maximal recovery.

### MHP36 grafts

MHP36 cells, examined ~6 months after grafting, were also clearly evident in Nissl stained sections in all four animals (Fig. 8). MHP36 grafts were of a consistent appearance, which differed from the two types of foetal graft, and exhibited two characteristic features. First, MHP36 cells formed diffuse aggregates around the site of injection. These aggregates were less compact than the tight swirls formed by foetal cells within the hippocampus and more integrated with the host tissue (cf. Figs 6 and 8B). Secondly, MHP36 cells migrated away from the site of transplantation and were found positioned throughout the lesioned CA1 field. MHP36 cells identified by antibodies to  $\beta$ -galactosidase (Figs 8 and 9A) and by autoradiography (Fig. 9B and C) were dispersed widely in the pyramidal CA1 field and the hippocampal



**Fig. 5** CA1 cells in control and lesioned marmosets. Sections at AP 4.5 mm (Stephan *et al.*, 1980) in the CA1 area used for counting cells (see Table 2). Sections on the left (A, C, E and G) show the greatest number of surviving host pyramidal cells and those on the right (B, D, F and H) show the smallest number of pyramidal cells from either hemisphere across all animals within each group. In controls (A and B) cell counts averaged 300 (range 254–327). One marmoset in the lesion-only (C and D) and two marmosets in the lesion plus CA1 grafted groups (E and F) showed good cell survival (to 50–80% of control level) in the hippocampus on one side of the brain, though in each case there were few or no surviving cells in the contralateral hippocampus. In marmosets with MHP36 grafts lesioning was almost complete; the maximum number of surviving pyramidal cells counted was 68. Within each of the lesion groups a zero score was recorded for at least half of the sections at this level. Scale bar = 100  $\mu$ m.

fissure. Other grafted MHP36 cells were found to have migrated into the adjacent white matter of the temporal cortex. In one animal that exhibited some dentate gyrus

damage,  $\beta$ -galactosidase labelled MHP36 cells were seen in the dentate granule layer.

Two approaches were used to identify individual cell

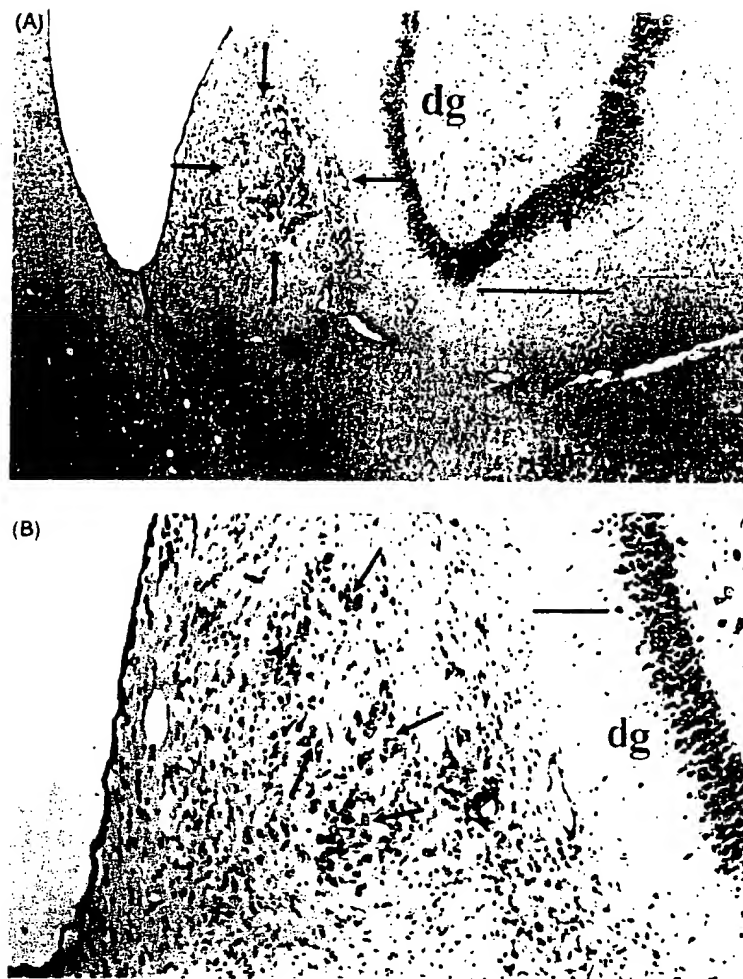


Fig. 6 Nissl-stained coronal sections showing a cluster of grafted foetal CA1 cells (arrows) within the lesioned host CA1 field. (A) Scale bar = 500  $\mu$ m. (B) At higher magnification ( $\times 100$ ) normal looking pyramidal cells (arrows) can be seen in the graft mass. dg = dentate gyrus; scale bar = 100  $\mu$ m.

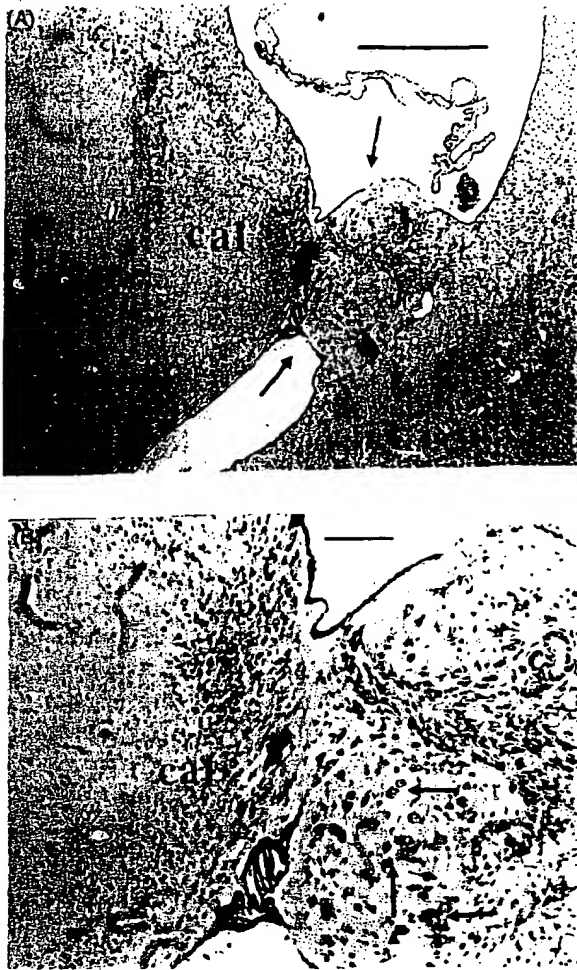
phenotypes of MHP36 grafts. First, serial sections were stained for  $\beta$ -galactosidase and  $\beta$ III-tubulin to identify neuronal phenotypes. The grafted areas proved positive for both  $\beta$ -galactosidase and  $\beta$ -tubulin indicating that some grafted cells express a neuronal phenotype. The same area stained positive for glial fibrillary acidic protein, but this was also seen in marmosets with lesions alone (Fig. 5C) and with foetal grafts, indicative of lesion-induced activated host astrocyte invasion. Secondly, examination of  $\beta$ -galactosidase labelled cells indicated differential morphology. Cells resembling classical neuronal or astrocytic phenotypes were seen near the transplantation sites (Fig. 8B). Cells that migrated away from the transplantation site and integrated into the CA1 field showed either a pyramidal or an astrocytic morphology (Fig. 9A). Cells which migrated into the white matter appeared to be exclusively astrocytic. In an animal with MHP36 cells in the dentate granule layer, the cells appeared to be solely neuronal and of granular appearance. Photomicrographs of graft histology can be viewed in colour at the ReNeuron web site (<http://www.reneuron.com/>).

## Discussion

### Lesion effects

The results showed that bilateral NMDA lesions of the CA1 field resulted in stable and long-lasting impairment both of retention of CDs first learned before lesioning, and of the ability to learn new discriminations. The finding that recall of SDs was not significantly disrupted suggests that the lesion induces a very specific cognitive impairment rather than having an effect on perception, motivation or motor function. Ridley and colleagues (Ridley *et al.*, 1995, 1997) have shown that CD tasks are disrupted by medial septal/diagonal band and fimbria–formix lesions, as well as by intrahippocampal damage, but not by entorhinal cortex lesions. The present results confirm these findings of a substantial selective disruption of acquisition and recall of CDs following damage within the hippocampus, and indicate that this disruption persists through a series of new acquisition tasks so that it may, in effect, be irreversible by time or practice. The only circumstances in which monkeys with hippocampal damage are unimpaired in CD tasks are (i) in retention of tasks





**Fig. 7** Nissl stained coronal sections showing a lobular foetal CA1 graft mass attached to the ventricular margin of the lesioned CA1 field. (A) The graft formed an artificial bridge to the white matter bordering the temporal cortex across the lateral ventricle. Scale bar = 500  $\mu$ m. (B) At higher power magnification ( $\times 100$ ) healthy looking grafted pyramidal cells (arrows) can be seen within the graft mass. Scale bar = 100  $\mu$ m.

learned (very slowly) in the lesioned state; and (ii) with repeated runs of the same stimuli during training (rather than a pseudorandom schedule), which may encourage learning of the task as separate SDs, rather than learning the conditional rule (Ridley *et al.*, 1996, 1997). Ridley and Baker argue that CD tasks require the ability to process different possibilities about the same stimuli simultaneously, rather than learning single invariant relationships serially (Ridley and Baker, 1997), conceptualized by Perner (Perner, 1991) as 'multi-modal' as opposed to 'single up-dating' information processing. This interpretation is consistent with previous suggestions that the hippocampus acts as a comparator specialized for processing 'relational' information (for a review, see Eichenbaum *et al.*, 1995). Findings that monkeys with septohippocampal system damage can only master CD tasks laboriously, indicate that this task normally requires a specialized type of encoding process for storage of information in long-term memory, for which the hippocampus

is critically important. Our findings indicate that CDs learned by the intact brain also require the hippocampus for retrieval (cf. Squire and Alvarez, 1995). However, a clear implication of graft-induced reversal of retention deficits is that information is not stored long-term within the hippocampus. Hippocampal-dependent CDs appear to be highly suitable for the investigation of graft effects following hippocampal damage, because they provide stable levels of impairment and potentially accessible memories against which to assess functional recovery over an extended period of testing.

### Foetal graft effects

The present results provide clear evidence of improvement both in the recall and learning of CDs following grafts of foetal tissue within the lesion sites. These results replicate the findings of Ridley and colleagues (Ridley *et al.*, 1997) in several respects. First, recall of discriminations was very substantially improved after foetal grafts in both studies. Secondly, learning of the first new CD task was only partially improved, hence graft-induced functional recovery appeared to be modest at this point. However, the present results show that if the animals are required to learn further discriminations, performance of marmosets with foetal grafts improves to control level and substantial recovery is evident. The failure of grafts to exert marked effects on cognitive performance when first tested could reflect incomplete integration of grafted cells into the host neural network and a longer interval may therefore be required for the development of full anatomical connectivity. Alternatively, grafted animals may require exposure to testing in order for functional integration to be forged by experience. In comparison, Mayer and colleagues found that rats with striatal grafts in unilateral striatal lesion sites required 2–3 weeks training before their reaction time to a contralateral light flash was normalized (Mayer *et al.*, 1992). Comparison of groups with and without prior experience would be necessary to elucidate whether time, training or these factors combined are critically responsible for recovery. Since impairment of lesioned animals was stable, it would also be possible to test animals early after transplantation to follow the time-course of recovery and to see whether this relates to the growth and connectivity of grafted cells. Within the small group of animals in the present experiment, there was no evidence that the eventual extent of functional recovery was related to specific features of the grafts, such as site, graft volume or number of transplanted cells, because all grafted animals showed maximal recovery. Grafts projecting haphazardly into the ventricles appeared to be just as effective as those which remained in the lesioned hippocampus. Anterior as opposed to posterior siting of grafts conferred no particular advantage. However, since bilateral CA1 lesions appear to be necessary to induce deficits in CD tasks (Ridley *et al.*, 1996), unilateral grafts might even be functionally effective in animals with bilateral lesions, and therefore correlations between graft

efficacy and number of grafted cells or graft volume would be difficult to establish.

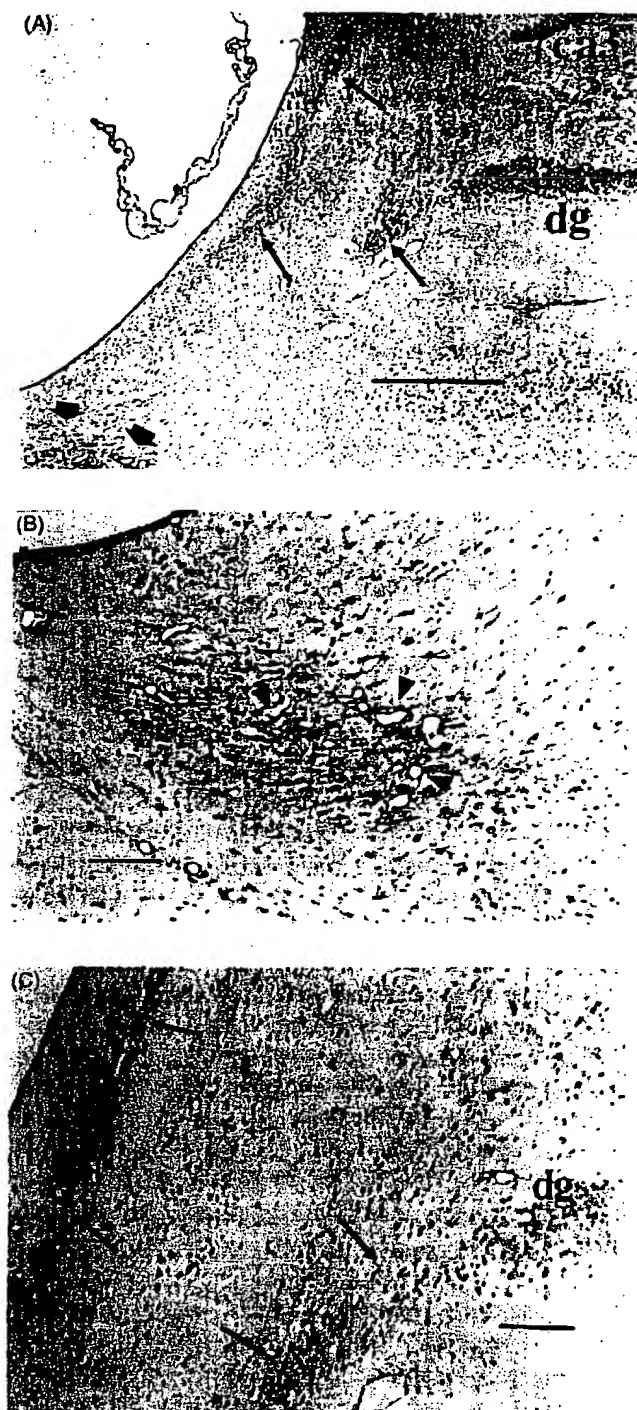
### MHP36 graft effects

The performance of marmosets with grafts of MHP36 clonal cells mirrored that of animals with foetal grafts throughout this experiment. Thus, marmosets with MHP36 grafts showed immediate improvement in recall of CDs, with partial improvement in learning the first new CD task, followed by full recovery to control level on subsequent learning of CD

tasks 2–4. However, animals with foetal grafts were first tested 12 weeks after transplantation, whereas those with MHP36 grafts were tested after only 6 weeks. This choice of time-points reflects previous studies of graft development in rodents, in which foetal hippocampal grafts in ischaemic-lesioned CA1 have been shown to establish connectivity by 8–10 weeks (Mudrick and Baimbridge, 1991) and MHP36 grafts have been shown to migrate to the area of ischaemic CA1 cell loss after 4 weeks (Sinden *et al.*, 1997). The similar functional recovery despite the discrepancy between intervals before testing suggests, however, that the passage of time may not be as important for graft efficacy as previous experience of the animals which enables them to 'learn to use' their grafts (Mayer *et al.*, 1992). Effects of MHP36 grafts in CA1-lesioned marmosets resemble those in rats with ischaemic (four vessel occlusion) damage to the CA1 field of the hippocampus, in which both foetal and MHP36 grafts improved spatial learning and spatial working memory to control level. However, given the derivation of MHP36 cells from transgenic mouse neuroepithelial stem cells, the functional efficacy of these cells in primate as well as rodent species breaks new ground and suggests that neuroepithelial stem cells possess remarkable plasticity and low immune response provocation.

### Graft histology

Histological examination indicated that foetal and MHP36 grafts exhibited very different patterns of host colonization. Foetal grafts were of two types. They either formed lobules attached to the ventricular margins of the CA1 field or they formed dense clusters within the hippocampus. No grafted foetal cell clusters were seen outside the hippocampus and lateral ventricles. These cell masses are typical of foetal grafts in rat hippocampus, although in the rat brain the corpus callosum above the CA1 field attracts more vigorous growth of foetal cells than the denervated CA1 field beneath (Hodges *et al.*, 1996). MHP36 grafts differed markedly from foetal grafts. Around the injection sites they formed diffuse



**Fig. 8** Coronal section through the CA1 lesion site stained for reactivity to  $\beta$ -galactosidase. (A) MHP36 cells migrating from a ventral injection site (bottom left, thick arrows) upwards through the CA1 field towards the CA3 junction. This region also stained positive for  $\beta$ III-tubulin, suggesting the presence of neurons within the graft (scale bar = 500  $\mu$ m). At higher magnification ( $\times 100$ ) the injection site (B) is well vascularized (arrowheads) and  $\beta$ -galactosidase positive cells appear more integrated than grafted CA1 cells (cf. Figs 6 and 7), and can be seen migrating upwards. Most of the cells near the injection site have an astrocytic appearance, but some show a neuronal phenotype. (C) MHP36 cells migrating towards the dorsal hippocampus in two broad streams: within the pyramidal cell layer heading towards CA3 and within the molecular layer heading towards the dentate gyrus (dg) which at this anterior level ( $\sim 7$  mm before the interaural line) is only just becoming visible. (Scale bars for B and C = 100  $\mu$ m.)



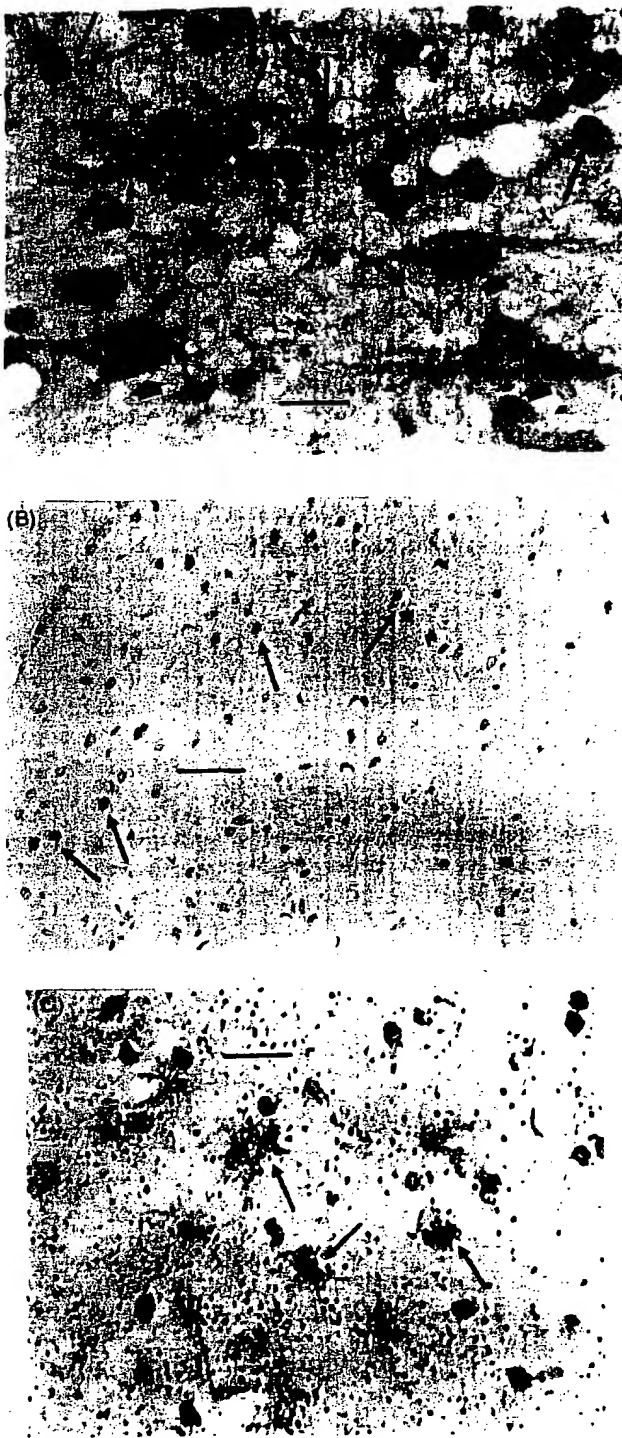


Fig. 9 (A) High power ( $\times 1000$ , scale bar =  $10\ \mu\text{m}$ ) magnification of  $\beta$ -galactosidase positive MHP36 cells at the border of the CA1 field and the temporal white matter. Some cells appear astrocytic (arrows) whilst others resemble pyramidal neurons (thick arrows), with the neuron-like cells in the CA1 field and astrocytic cells moving into the white matter. (B) Autoradiograph of [ $^3\text{H}$ ]thymidine labelled MHP36 cells show their relatively even and sparse distribution in the lesioned CA1 field, which contrasts with the clustering (cf. Fig. 6) of foetal CA1 cells (scale bar =  $100\ \mu\text{m}$ ). (C) Double labelling of cells with  $\beta$ -galactosidase and [ $^3\text{H}$ ]thymidine positive cells (arrows) confirms that these markers identify grafted MHP36 cells (scale bar =  $10\ \mu\text{m}$ ).

aggregates which were more integrated into the host brain than the compact masses formed by foetal grafts. Unlike foetal cells, which remained in discrete clumps, MHP36 cells migrated away from the site of transplantation and were distributed throughout the lesioned CA1 field. This pattern of migration was surprisingly similar to that shown by MHP36 cells in ischaemic rat brain. In the rat, MHP36 cells migrate to the damaged CA1 layer where, to a lesser or greater extent, they form a compact and dense alignment of pyramidal-like cells, resembling the normal CA1 field (Sinden *et al.*, 1997). In marmosets the CA1 field is broader and less densely packed, which is characteristic of primate brain. However, with their even and widespread distribution MHP36 cells appeared to reconstruct the normal appearance of this field, as in the rat. MHP36 cells have been seen to migrate into the rat dentate granule layer damaged by needle penetration, just as they were found in one marmoset with dentate gyrus damage. As in the rat, MHP36 cells in the CA1 field showed both neuronal pyramidal-like and astrocytic types. Findings that MHP36 cells in white matter were astrocytic, whereas those in the dentate gyrus were neuronal and of granular appearance, support the evidence from other laboratories that differentiation of precursor stem cells is site-specific (Gage *et al.*, 1995; Shihabuddin *et al.*, 1996). However, our finding that MHP36 cells differentiated within discrete regions of brain damage contrasts with the findings of Gage and colleagues with FGF-responsive hippocampal progenitor cells (Gage *et al.*, 1995), and Shihabuddin and colleagues with the RN33B cell line cloned from medullary raphe progenitor cells. They found that grafts derived from stem cells develop host-type neuronal morphologies only when transplanted into unlesioned sites and not, as we found, lesioned sites (Shihabuddin *et al.*, 1996).

### Conclusions

In conclusion, the present findings confirm that NMDA lesions of the marmoset hippocampal CA1 field induce long-lasting and profound impairment in retention of CDs first learned before lesioning, and in acquisition of new discriminations after lesioning, but have negligible effects on recall of SDs. These results suggest that lesions induced specific cognitive, but not motor, perceptual or motivational impairment. Grafts of foetal CA1 tissue and of conditionally immortalized clonal MHP36 cells derived from the H-2K<sup>b</sup>-tsA58 transgenic mouse neuroepithelium were equally effective in reversing lesion-induced deficits. Recall of CDs was improved to control level on first testing after transplantation. Impairment of grafted animals in learning the first, but not subsequent CDs suggested that time and/or training may be important for functional graft recruitment. Gross histological examination indicated different patterns of innervation with foetal and MHP36 grafts. Foetal grafts formed lobules attached to the ventricular margin of the CA1 field, or clumps within the CA1 lesion sites. Grafted MHP36 cells were evenly distributed in the denervated host CA1 field

where they adopted both neuronal and astrocytic phenotypes. These results suggest that both foetal and MHP36 grafts promote recovery from damage to the hippocampus, and that this may involve partial reconstruction of hippocampal circuitry. However, in view of the very different patterns of foetal and MHP36 cell distribution, the precise mechanisms by which foetal and MHP36 grafts exert their functional effects may differ; further work is needed to determine factors which govern patterns of migration and to characterize grafted MHP36 cell types. Nevertheless, this first demonstration of robust improvement with a conditionally immortalized cell line in a primate species offers a promising prospect for the development of non-foetal grafts to alleviate the effects of circumscribed brain damage.

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## Exhibit D

A human pluripotent neuroepithelial cell line was developed by means of culturing primary human fetal cortex of 12 weeks gestation obtained from elective termination under a London Teaching Hospital Ethical Committee approved protocol and following guidelines set out in the Polkinghorne Report (Review of the Guidance on the Research Use of Foetuses and Foetal Material presented to Parliament July 1989, London, HMSO). The primary cells were plated on laminin-coated flasks and infected after attachment with an amphotropic virus encoding the temperature-sensitive SV40 tsA58-U19 mutation gene. Following 4 weeks of expansion at the gene-permissive temperature (33° C), several colonies of Tag-positive cells emerged. Following cell purification, clones were ringed, picked and expanded. One of these lines, RCB5600OGCX (herein abbreviated to CX), was found to be positive for a range of neuroepithelial stem cell markers, including nestin and musashil. This line was conditional to temperature, showing growth at 33° C, but no growth and partial differentiation at 37° C. It was confirmed as clonal by Southern blotting, with a single integration site for the tsTAg gene.

Effects of the human conditionally immortal clonal cell line (CX) were assessed in rats with unilateral basal forebrain excitotoxic lesions of the cholinergic projections from basal forebrain to cortex. This lesion mimics some of the cell loss that occurs in Alzheimer's disease and other neurodegenerative conditions, and gives rise to robust deficits in learning and memory tasks, including spatial learning in the water maze. The water maze task requires rats to find a submerged platform located in the center of one quadrant in a large (200 cm diameter) circular swimming pool.

In the first study (AD5), effects of the human line were assessed in comparison with several murine cell lines, including MHP36 reported here as a positive control, and with sham-grafted lesioned and non-lesioned controls. Rats received unilateral injections of AMPA (0.3 µl in two sites: rostral and caudal) into the basal forebrain nucleus basalis magnocellularis (nbm). Non-lesioned controls received vehicle at the same sites. Twenty-one days after lesioning, rats received unilateral cell suspension or sham vehicle grafts (n per group = 10) into the frontal and parietal cortex, the terminal regions of cholinergic basal forebrain projections. These sites have been associated with substantial

graft-induced behavioral recovery in this lesion model. Eight weeks later, all groups were trained to find a submerged platform in a water maze test where poor performance across several parameters (latency to reach the platform, distance swum and time spent in appropriate sectors) reflects spatial long- and short-term learning and memory impairments. The lesion-only group showed little improvement over 14 days, taking 45 seconds to reach the platform by the end of training, compared with c. 15 seconds by controls. Rats grafted with the murine MHP36 cell line performed significantly better than lesioned animals. However, rats receiving the human cell line (CX) showed as rapid spatial learning as controls, and were superior both to the lesion and the murine grafted groups. On the probe trial, with the platform removed, which tests memory for its location by time spent searching in the appropriate sector, controls and rats grafted with human and murine cell lines spent 40%-50% of time in the platform quadrant, showing comparable good recall of its position, whereas lesion-only rats spent 25% of time there, showing chance level recall. The superiority of human and murine grafted groups relative to lesion-only animals was seen also in a working memory task of rapid spatial learning, when the position of the platform was changed each day.

In order to replicate the substantial positive effects of the human cell line 500 CX, a second study (AD6) was carried out comparing grafted rats with sham-grafted lesioned and non-lesioned controls. Lesion, grafting and testing parameters were the same as those in the previous experiment (AD5) with the exception that rats were tested at six rather than eight weeks after grafting. Results of the two experiments were highly comparable. Spatial learning in grafted rats was as efficient as in controls in all parameters; probe trial performance showed good recall of the platform position in control and grafted groups and poor memory in lesioned rats, whilst working memory in control and grafted groups was also superior in control and grafted animals relative to the lesion-only group. These results confirmed that grafts of a human cell line were able to promote recovery to control level in rats that showed marked deficits in spatial learning and memory following substantial (80%-90%) unilateral depletion of forebrain acetylcholine.

## Nestin-EGFP Transgenic Mice: Visualization of the Self-Renewal and Multipotency of CNS Stem Cells

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We generated transgenic mice carrying enhanced green fluorescent protein (EGFP) under the control of the *nestin* second-intronic enhancer (E/*nestin*:EGFP). Flow cytometry followed by *in vitro* assays revealed that *in situ* EGFP expression in the embryonic brain correlated with the mitotic index, the cogeneration of both neurons and glia, and the frequency of neurosphere formation *in vitro*. High-level EGFP expressors derived from embryos included a distinct subpopulation of cells that were self-renewable and multipotent, criteria that define neural stem cells (NSCs). Such cells were largely absent among lower-level or non-EGFP expressors, thereby permitting us to enrich for NSCs using EGFP expression level. In adults, although E/*nestin*:EGFP-positive cells included the NSC population, the frequency of neurosphere formation did not correlate directly with the level of EGFP expression. However, moderately EGFP-expressing cells in adults gained EGFP intensity when they formed neurospheres, suggesting embryonic and adult NSCs exist in different microenvironments *in vivo*.

### INTRODUCTION

During mammalian development, stem cells play a critical role in histogenesis and organogenesis, and in the maintenance of the resulting structures by providing the phenotypically restricted progenitors from

which differentiated phenotypes arise. The biology of stem cells, especially the molecular mechanisms that regulate their generation, maintenance, and differentiation, is best studied using methods in which the behaviors of isolated or enriched populations of stem cells can be analyzed under carefully controlled conditions (Morrison *et al.*, 1997, 1999; Barres, 1999). For such studies, the ability to perform “prospective identification,” that is, to identify live stem cells in dissociated native tissue, is crucial. In the central nervous system (CNS), however, the lack of surface antigens specific to neural stem cells (NSCs) prohibits applying cell sorting techniques to the study of NSCs; as a result, NSCs have not yet been readily identifiable from mammalian brain tissue. Intriguingly, Johansson *et al.* (1999) found that ependymal cells in the adult brain can be isolated based on Notch1 expression, and that these cells exhibit the cellular properties of stem cells *in vitro*. Doetsch (1999) used different methodologies to conclude that at least some subependymal astrocytes may be adult NSCs. Yet although provocative, neither of these studies involved the prospective identification of NSCs. The need to further understand these intriguing results has led in turn to a critical need for a means to locate, identify, and select live CNS stem cells.

To achieve this end, we used green fluorescent pro-

tein (GFP), placed under the transcriptional control of the neural-specific second intronic enhancer of the *nestin* gene (Zimmerman et al., 1994), as a live-cell reporter of the neural progenitor phenotype. Nestin is an intermediate filament transiently expressed during neural ontogeny; in development, it is expressed first by neuroepithelial cells and radial glia, and later by progenitor cells of the ventricular zone (during the embryonic stage) and the nascent ependyma/subependyma (during the postnatal stage) (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990; Doetsch et al., 1997; Lothian and Lendahl, 1997). Using a variety of neural cell markers, we recently found that a subset of embryonic brain cells shows a particularly high level of immunoreactivity for Nestin, as well as for

the neural RNA binding protein, Musashi-1 (Kaneko et al., 2000). This observation suggested that Nestin might be expressed at different levels among different classes of CNS progenitor cells, despite its seemingly ubiquitous expression by the embryonic neuroepithelium. Furthermore, among the marker molecules reported to label CNS progenitor cells, only the *nestin* gene has a cis-element, within its second intron, that is well characterized and available for expressing foreign genes *in vitro* and *in vivo* (Zimmerman et al., 1994; Lothian and Lendahl, 1997).

Based on these facts, we postulated that if we could drive GFP expression using the cis regulatory elements for *nestin*, we could fractionate progenitor cells based on the intensity of the GFP fluorescence. We have already

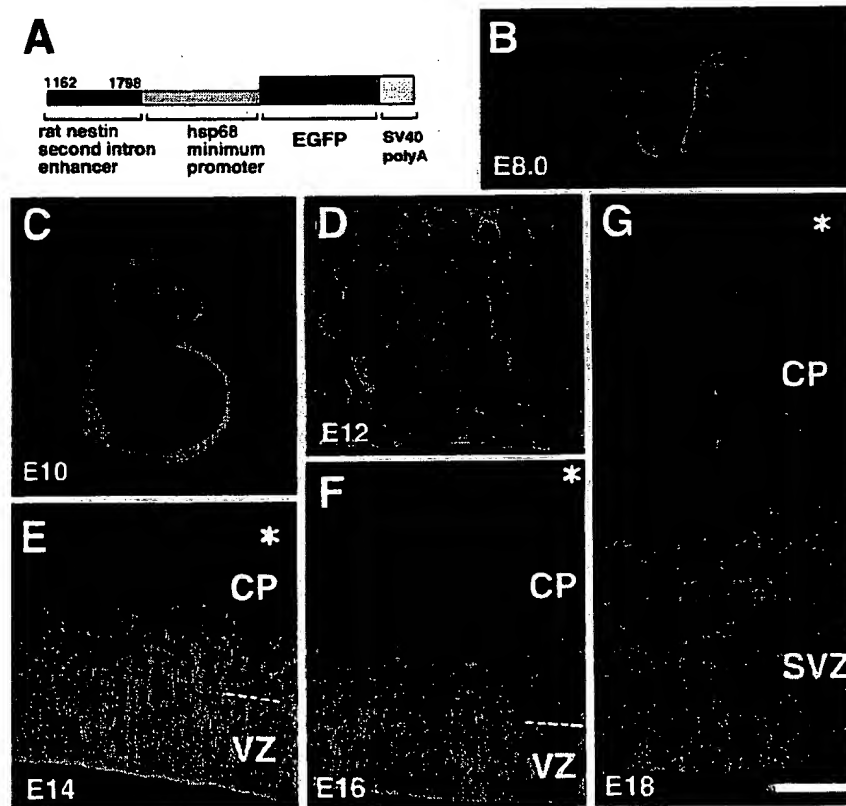


FIG. 1. E/*nestin*:EGFP-transgenic mice. (A) Structure of the E/*nestin*:EGFP transgene. The second intron enhancer of the rat *nestin* gene (1162–1798) (Lothian and Lendahl, 1997) was placed upstream of the minimum promoter of heat shock protein 68 (*hsp68*) (Rossant et al., 1991) fused to EGFP cDNA and a polyadenylation signal. (B) Section of an E8.0 embryo, showing EGFP expression restricted to the neural plate. (C) Whole-mount fluorescent micrograph showing EGFP expression throughout the CNS of an E10 embryo. (D–G) Section of the cerebral wall at E12 (D), E14 (E), E16 (F), and E18 (G). At E12, EGFP fluorescence was detected from the ventricular to the pial surface of the cerebral wall, but the overall level of EGFP expression was lower than at E14–18. At E14–18, EGFP was predominantly expressed in the ventricular zone (VZ), and not in the cortical plate (CP). Asterisk: pial surface. CP, cortical plate; VZ, ventricular zone; SVZ, subventricular zone. Bar: 80  $\mu$ m in B; 1.3 mm in C; 27  $\mu$ m in D; 90  $\mu$ m in E–G.





**FIG. 2.** Immunocytochemical characterization of EGFP-expressing cells. Forebrain cells harvested from E14–15 E/nestin:EGFP mice were doubly immunostained with anti-GFP (A, D, G, J, L) and anti-Nestin (Rat401) (B), anti-Musashi1 (E), RC2 (H), anti- $\beta$ III-tubulin (K), or anti-PSA-NCAM (M) antibodies. Both EGFP<sup>+</sup> cells (arrowhead in A) and EGFP<sup>2+</sup> cells were Nestin<sup>+</sup> (B). Musashi1 immunoreactivity was detected in EGFP<sup>2+</sup> cells, but also in some EGFP<sup>+</sup> cells (dashed arrow in D–F). RC2 immunoreactivity was almost completely confined to EGFP<sup>2+</sup> cells (G, H).  $\beta$ III-tubulin was detected in some EGFP<sup>+</sup> cells but in no EGFP<sup>2+</sup> cells (arrows, J, K). EGFP<sup>2+</sup> cells were also PSA-NCAM negative (double-arrowheads, L, M). Bar, 50  $\mu$ m in A–F, 20  $\mu$ m in G–M.

used *nestin* second intronic enhancer-driven EGFP (E/nestin:EGFP) to identify neuronal or neural progenitor cells in both the adult human periventricular area and hippocampus (Roy *et al.*, 2000a,b). However, these experiments were performed by transfecting dissociated brain cells with E/nestin:EGFP plasmid DNA. As a result, the intensity of GFP-fluorescence, and hence the yield of identified progenitor cells, likely varied as a

function not only of the transfection efficiency ( $9.40 \pm 0.9\%$ , mean  $\pm$  SEM) (Roy *et al.*, 2000b), but also of the plasmid copy number in each successful transfectant. In addition, the transient nature of plasmid transfection precluded the use of this technique for the long-term observation and monitoring of sorted progenitor cells, whether *in vivo* or *in vitro*.

To address these concerns, we took a transgenic approach towards fractionating neural progenitor cells on the basis of *nestin* transcription. To this end, we established transgenic mice in which *nestin* enhancer-driven transcriptional activation can be reported by EGFP expression. In the embryonic brains of these transgenic mice, cells expressing a high level of EGFP are largely limited to the ventricular zone (VZ). Using flow cytometry combined with the *in vitro* examination of clonal or neurosphere cultures of sorted cells, we found that the activation of the *nestin* enhancer is highest in cells that can potentially proliferate, self-renew, and generate both neurons and glia, which are all criteria that define NSCs. Thus, this mouse line provides a useful tool with which to monitor these developmental characteristics in culture and assess them *in vivo*.

## RESULTS

### EGFP Expression was Restricted to the Ventricular Zone in E/nestin:EGFP-Transgenic Mice

Transgenic mice expressing EGFP under the control of the rat *nestin* second intronic enhancer were generated (Fig. 1A). We obtained six independent lines of E/nestin:EGFP transgenic mice that showed indistinguishable patterns of EGFP expression in the developing CNS, indicating the consistent expression profile of this transgene (Fig. 1A).

By microscopic examination, the EGFP expression was detectable as early as E7. At this stage, the anti-GFP immunoreactive cells were sporadically distributed in the one-cell-thick neural ectoderm (data not shown). At E8, EGFP expression was readily detected in the neural plate (Fig. 1B). At E10, EGFP fluorescence was seen throughout the CNS (Fig. 1C).

We further examined the stage-dependent changes in the expression pattern of EGFP, focusing on the cerebral wall where the distribution of proliferative and differentiated cells has been extensively studied (Smart, 1983; Takahashi *et al.*, 1995). At E12 (Fig. 1D), EGFP expression was detected in the ventricular zone (VZ), but both the overall and highest expression levels were lower than at E14 (Fig. 1E) and later (Figs. 1F and 1G). During

the period of active neuronogenesis and formation of the cortical plate (CP) (Figs. 1E and 1F), a high level of EGFP expression was seen in the ventricular side containing the VZ, and the fluorescence was strongest along the ventricular surface. At E18, EGFP expression in the VZ had become sparse, but was still highest along the ventricular side (Fig. 1G).

### Strongly E/nestin:EGFP-Positive Cells Expressed Progenitor Markers, But No Mature Neural Markers

We next examined the antigenic phenotypes of brain cells categorized on the basis of their E/nestin:EGFP expression. Microscopic observation of cerebral cells dissociated from E14–15 mice revealed three categories of cells with respect to their EGFP levels: negative (EGFP<sup>-</sup>, a level indistinguishable from that of cells derived from wild-type mice), weakly positive (EGFP<sup>+</sup>), and strongly positive (EGFP<sup>2+</sup>). EGFP<sup>2+</sup> cells were three to five times brighter than EGFP<sup>+</sup> cells when measured by Photoshop (Adobe Systems). About 25% of the total cerebral wall cells at E14 were EGFP<sup>2+</sup>.

All E/nestin:EGFP-expressing cells (both EGFP<sup>2+</sup> and EGFP<sup>+</sup>) expressed Nestin (Figs. 2A–2C), and Musashi-1, another antigenic marker of neural progenitor cells, including NSCs (Sakakibara et al., 1996; Kaneko et al., 2000) (Figs. 2D–2F) (Table 1). Immunoreactivity for RC2, which is selectively expressed by radial glia and neural progenitor cells (Misson et al., 1988; Kaneko et al., 2000), was detected in EGFP<sup>2+</sup> cells, of which about 70% were RC2<sup>+</sup> (Figs. 2G–2I) (Table 1). Conversely, RC2<sup>+</sup> cells almost invariably expressed both E/nestin:EGFP fluorescence and Musashi-1 immunoreactivity. Thus, RC2-defined radial cells comprised a subset of E/nestin:EGFP-positive ventricular zone cells.

In general, E/nestin-driven EGFP expression was more robust in undifferentiated progenitor cells than in their differentiated daughters: EGFP<sup>2+</sup> cells failed to express neuronal  $\beta$ III-tubulin (Figs. 2J and 2K) or MAP2 (Table 1), astroglial GFAP, or oligodendroglial O4 (not shown). The EGFP<sup>2+</sup> cells also failed to express detectable PSA-NCAM (Seki and Arai, 1991) (Figs. 2L and 2M) (Table 1). Notably, some EGFP<sup>+</sup> cells were  $\beta$ III-tubulin-positive (Figs. 2J and 2K), suggesting that phenotypic maturation was accompanied by down-regulation of the E/nestin:EGFP expression.

### Time-Lapse Observation of the Cytogenesis of Embryonic EGFP<sup>2+</sup> Cells

We further assessed the lineage potentials of EGFP<sup>2+</sup> cells *in vitro*. First, cerebral cells dissociated from E14 transgenic embryos were plated onto polyethylenimine (PEI)-coated plastic dishes at a low density, and EGFP<sup>2+</sup> cells ( $n > 50$ ) were chosen upon the initial microscopic observation (<2 h after plating). The behavior of each chosen cell was then time-lapse recorded.

About 50% of the total EGFP<sup>2+</sup> cells observed divided within 24 h, and the remaining EGFP<sup>2+</sup> cells died. Figure 3A shows the generation of two daughter cells from a single EGFP<sup>2+</sup> cell, in which one of the daughter cells expressed a lower level of EGFP (indicated by a single arrowhead) and was  $\beta$ III-tubulin<sup>+</sup>, while the other retained a high level of EGFP expression (double-arrowheads in Fig. 3A) and was  $\beta$ III-tubulin negative. Clones generated by single EGFP<sup>2+</sup> cells by 1 day in culture often (about 50%) contained cells that were different, both in their morphology and expression of molecular markers.

In longer (>3 day) culture, most (60–80%) clones that were formed by single EGFP<sup>2+</sup> cells contained both neurons ( $\beta$ III-tubulin<sup>+</sup>) and astrocytes (GFAP<sup>+</sup>) that did not show (or only faintly showed) EGFP fluorescence (Fig. 3B). These observations indicate that (1) the intensity of EGFP fluorescence may reflect cells' developmental status or potential, and that (2) EGFP<sup>2+</sup> cells can divide to generate both neurons and glia *in vitro*.

To further assess the ability of the EGFP-expressing cells to continue to proliferate, we used neurosphere cultures (Reynolds and Weiss, 1992, 1996). In the presence of EGF and/or bFGF, CNS stem cells proliferate in a self-renewing manner to generate cell aggregates, or spheres, containing mostly undifferentiated cells. Time-lapse recording of sphere formation from single EGFP<sup>2+</sup> cells (Fig. 4A) demonstrated that newly generated cells showed EGFP fluorescence at a level similar to that of

TABLE 1  
Antigen Expression of EGFP<sup>2+</sup> Cells from E14 Embryos

$\alpha$ -Nestin	100%
$\alpha$ -Musashi1	100%
RC2	69.2 $\pm$ 8.6%
$\alpha$ - $\beta$ III-tubulin	0%
$\alpha$ -MAP2	0%
$\alpha$ -PSA-NCAM	0%

Note. These cells expressed progenitor but not neuronal markers. Data indicate the mean  $\pm$  SD from 3 samples (>200 EGFP<sup>2+</sup> cells in each sample).

the sphere-initiating cells during the first 2–3 days, until the sphere became too large for analysis at the single-cell level. By 7 days, highly fluorescent spheres had formed. More than 90% of the cells within these "bright" spheres coexpressed E/nestin-driven EGFP (EGFP<sup>+</sup> or EGFP<sup>2+</sup>) and Nestin protein (*not shown*). Of the total cells dissociated from these spheres, 50–70% were EGFP<sup>2+</sup>. Interestingly, RC2<sup>+</sup> immunoreactivity was also detected in 50–70% of the dissociated cells (*not shown*), consistent with our previous report that Musashi-1<sup>+</sup>/Nestin<sup>+</sup>/RC2<sup>+</sup> cells comprised the largest antigenic phenotype in spheres (Kaneko *et al.*, 2000). Since the proportion of EGFP<sup>2+</sup> cells in the embryonic cerebral cells before culturing was about 25% *in vivo* at E14, the sphere culture resulted in a greater enrichment in the EGFP<sup>2+</sup> cell population (50–70%).

Bright spheres transferred onto PEI-coated dishes formed cellular sheets within several days (Fig. 4B). At the thinnest (most well spread) part of the bright sphere-derived sheets, EGFP fluorescence was lost. In these areas,  $\beta$ III-tubulin<sup>+</sup> neurons, GFAP<sup>+</sup> astrocytes, and O4<sup>+</sup> oligodendrocytes were observed (Fig. 4C). This result indicates that the sphere-initiating EGFP<sup>2+</sup> cells were multipotent.

Although the intensity of EGFP fluorescence in monolayer cultured cells seemed to be affected to some degree by cell shape (Fig. 3B), these *in vitro* results overall suggest that the EGFP expression levels correlate with the developmental potentials, as well as the antigenic phenotypes, of E14 cerebral cells *in vitro*. Therefore, this transgenic mouse line may be useful to infer in real time these important aspects of cellular functions during development.

#### ***E/nestin:EGFP-Based FACS Yields a Population of Nestin<sup>+</sup>/ $\beta$ III-Tubulin<sup>-</sup> Cells***

The preceding observations suggested to us that as a population, E/nestin:EGFP-positive cells included multipotential neural progenitor cells. To determine directly the correlation between EGFP expression and cell properties, and whether cells displaying the highest level of EGFP fluorescence fulfill the definitional criteria for NSCs, we subjected embryonic E/nestin:EGFP-transgenic brain cells to fluorescence-activated cell sorting (FACS).

Dissociated E14 murine brain cells were sorted into one of three fractions, each defined by its EGFP-fluorescence intensity as measured by the FL1 photomultiplier of the cell sorter (Fig. 5). These groups were defined so that the numbers of cells gated into the three fractions (F<sup>-</sup>, F<sup>+</sup>, and F<sup>2+</sup>; Fig. 5) were 30, 60, and 10% of

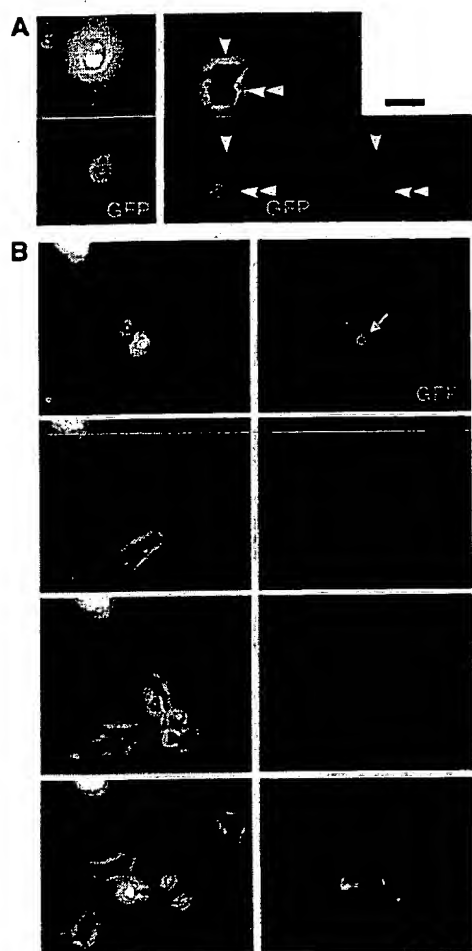
the total number of cells applied, respectively. The proportions of sorted cells that were optically judged as EGFP<sup>2+</sup> (determined by live examination and immunochemically) were >95% in F<sup>2+</sup>, 20–25% in F<sup>+</sup>, and <1% in F<sup>-</sup>.

First, we characterized the cells in these three fractions using the antibodies against Nestin protein and  $\beta$ III-tubulin. As expected, Nestin<sup>+</sup> cells were most abundant in F<sup>2+</sup> with a proportion of  $98.2 \pm 0.3\%$  (mean  $\pm$  SD), and the proportions of Nestin<sup>+</sup> cells were lower in F<sup>+</sup> and F<sup>-</sup> ( $68.0 \pm 15.7\%$  and  $24.9 \pm 14.4\%$ , respectively) (Table 2A). The level of Nestin immunoreactivity per positive cell also tended to be lower in the F<sup>-</sup> group. The proportions of  $\beta$ III-tubulin<sup>+</sup> cells showed a reciprocal pattern to those of Nestin<sup>+</sup> cells:  $0.9 \pm 0.3\%$  in F<sup>2+</sup>,  $37.5 \pm 7.7\%$  in F<sup>+</sup>, and  $69.8 \pm 12.0\%$  in F<sup>-</sup> (Table 2A). Both Nestin and  $\beta$ III-tubulin were simultaneously expressed in some cells sorted into F<sup>+</sup> (20–30%) and F<sup>-</sup> (10–20%), probably reflecting a transition in the expression of these proteins during neuronal differentiation (Frederiksen and McKay, 1988; Kaneko *et al.*, 2000). These data indicated that E/nestin-driven EGFP fluorescence intensity correlated with Nestin protein expression and that both were negatively associated with neuronal antigenic maturation.

#### ***E/nestin:EGFP<sup>2+</sup> Ventricular Zone Cells Are Mitotic***

To assess the mitotic competence of those cells having the highest level of EGFP-fluorescence, we next determined whether they were proliferating *in vivo*. Forebrain cells were isolated from E14 embryos whose mothers had been given BrdU twice during pregnancy (40 and 10 min before hysterotomy). The cells were then sorted by FACS, plated, and immunostained shortly (<1 h) thereafter. The resulting BrdU-labeling indices (Table 2B) suggested that those cells exhibiting the strongest transcriptional activation of the *nestin* enhancer were the most likely to be at S phase as a population at this embryonic stage.

We next examined whether E/nestin:EGFP<sup>2+</sup> cells were mitotic *in vitro* as well as *in vivo*. Sorted E15 forebrain cells were plated onto PEI-coated coverslips, cultured 12 h in DMEM/F12-based serum-free media with EGF and bFGF (20 ng/ml each), then treated for 1 h with BrdU (10  $\mu$ M), fixed, and stained with anti-BrdU. The BrdU-labeling index of the F<sup>2+</sup> cell fraction (33.7%) contrasted sharply with that of F<sup>-</sup> cells (3.1%) (Table 2B), indicating that, under these culture conditions, cells with the strongest EGFP fluorescence were selectively mitotic relative to fetal brain cells in which *nestin* transcription was no longer active.



**FIG. 3.** Developmental potentials of embryonic EGFP<sup>+</sup> cells in monolayer culture. Time-lapse observation of the differentiation of a single EGFP<sup>+</sup> cell of the E14 cerebral wall in monolayer culture. The number in each pair of phase-contrast and fluorescent pictures indicates the day in culture. (A) A single EGFP<sup>+</sup> cell divided by 1 day to produce two daughter cells showing different levels of EGFP fluorescence. One of them expressed a lower level of EGFP fluorescence (arrowhead) and was positive for the neuronal marker  $\beta$ III-tubulin<sup>+</sup>, while the other cell expressed a higher level of EGFP (double-arrowheads) and was  $\beta$ III-tubulin<sup>+</sup>, indicating that this cell division was asymmetric. (B) A single EGFP<sup>+</sup> cell (arrow) produced neurons and astrocytes in the monolayer culture. During differentiation of the cell, EGFP fluorescence intensity decreased. By day 3, the EGFP fluorescence had decreased to a nondetectable level, and the clone consisted of  $\beta$ III-tubulin<sup>+</sup> neurons (green) and GFAP<sup>+</sup> astrocytes (red). Bar: 20  $\mu$ m in A, 50  $\mu$ m in B.

#### **EGFP<sup>+</sup> Cells Generate Neurons and Astrocytes in Low-Density Monolayer Culture**

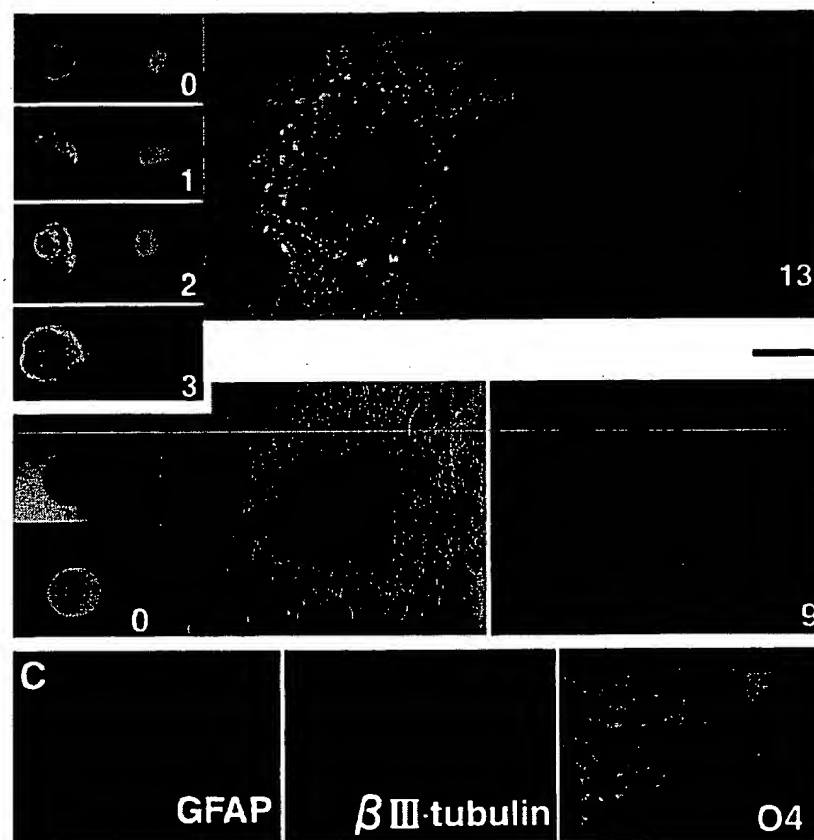
To assess the lineage potential of the sorted cells from the E14 forebrain, we cultured them on PEI-coated plas-

tic dishes at a low density (750 cells/cm<sup>2</sup>) for 7 days. Clones formed by these cells were categorized into three groups according to immunocytochemical results (Table 2C): (1) clones consisting of  $\beta$ III-tubulin<sup>+</sup> neurons only ("N" clones), (2) clones having GFAP<sup>+</sup> astrocytes but not neurons ("A" clones), and (3) clones having both neurons and astrocytes ("N + A" clones). "N + A" clones were found in F<sup>-</sup>, F<sup>+</sup>, and F<sup>2+</sup> cultures; however, the frequency of their formation was highest in the F<sup>2+</sup> (79.2%,  $n = 221$  clones), compared with F<sup>+</sup> (30.0%,  $n = 135$ ), and F<sup>-</sup> (15.5%,  $n = 71$ ) (Table 2C). However, these values were affected by differences in plating efficiency between each fraction. We thus calculated the frequency of cells that survived to form N + A clones per plating by multiplying the frequency of N + A clones by the respective plating efficiency of each fraction (Table 2C). This value (F<sup>-</sup>:F<sup>+</sup>:F<sup>2+</sup> = 1:2.9:20) might represent a correlation between the EGFP-fluorescence intensity and the lineage potentials of the sorted cells. Taken together, these data indicate a preferential enrichment of multipotent progenitor cells in the pool of the highly E/nestin:EGFP-expressing cells.

#### **FACS-Sorted E/nestin:EGFP<sup>+</sup> Cells Self-Renew and Generate Neurons, Astrocytes, and Oligodendrocytes through Primary and Long-Term Sphere Cultures**

**Primary sphere formation and differentiation assay.** We next examined the ability of the sorted cells to form spheres, and further tested whether the cells that showed the highest level of EGFP were multipotent and self-renewing, which are criteria for NSCs. E14 cerebral cells were fractionated by FACS, and the viable cells in each fraction were counted and plated at a density of 10<sup>4</sup> cells/ml. These cells were cultured for 7 days in serum-free media supplemented with 20 ng/ml each of FGF2 and EGF, then the number of neurospheres with a diameter >50  $\mu$ m was counted.

We found a striking difference between the strongly E/nestin:EGFP-expressing cells and the nonfluorescent controls in the number of sphere-initiating cells harbored: Whereas 3.37% of F<sup>2+</sup> cells generated spheres, only 0.42  $\pm$  0.05% of the sorted but unfractionated controls did so (Fig. 6). Both of these proportions may have been artifactually low, because sphere formation appeared to be impeded by the relatively traumatic process of cell sorting: Sphere formation by sorted but unfractionated control cells (0.42  $\pm$  0.05%) was only one-sixth that of unsorted controls (2.52  $\pm$  0.31%, mean  $\pm$  SD,  $n = 4$  wells), even though the cells were viable at the time of plating. Thus, the sorting proce-



**FIG. 4.** Embryonic EGFP<sup>+</sup> cells form neurospheres that are enriched with EGFP<sup>+</sup> cells. (A) A series of time-lapse photomicrographs showing the formation of a fluorescent sphere from a single EGFP<sup>+</sup> cell (the identical field is shown). The number in each pair of phase-contrast (left) and fluorescent (right) pictures indicates the day in culture. The observed field was identified using a scratch on the culture dish as a reference (not shown). (B) An EGFP fluorescent sphere, which was generated from a single EGFP<sup>+</sup> cell, was transferred onto a PEI-coated dish (left), where it formed a cellular sheet by day 9 after transfer (right). Cells in the periphery of the sheet, which had spread thin, forming a monolayer, were EGFP-negative. In contrast, cells in the thicker part (the center) of the sheet still expressed EGFP. (C) In cellular sheets formed by clonally cultured spheres that were initiated by single EGFP<sup>+</sup> cells, GFAP<sup>+</sup> astrocytes, βIII-tubulin<sup>+</sup> neurons, and O4<sup>+</sup> oligodendrocytes were generated, indicating the multipotency of the sphere-initiating EGFP<sup>+</sup> cells. Bar: 25 μm in A; 210 μm in B; 20 μm in C.

dures itself may have been detrimental to cell survival and/or proliferation. Accounting for the roughly 1/6 recovery by FACS of neurosphere-competent stem cells, one might predict that the frequency of sphere-initiating cells in F<sup>2+</sup> was approximately 20.0% ( $3.37 \times 6$ ) at E14 (Fig. 6).

We next examined the lineage potential of those sphere-initiating cells enriched in the F<sup>2+</sup> fraction. To this end, we transferred spheres formed in F<sup>2+</sup> cultures to PEI-coated coverslips, and cultured them as monolayers for 7–10 days. Of 27 single sphere-derived sheets, 25 (93%) developed various proportions of neurons, astrocytes, and oligodendrocytes together; 2 (7%) contained only neurons and astrocytes. Thus, cells strongly

expressing E/nestin-driven EGFP (F<sup>2+</sup> fraction) included sphere-initiating progenitor cells that were multipotent, which represented at least 20.0% of the cells in this fraction.

**Secondary sphere culture to examine the self-renewal of the sorted cells.** To examine the capacity of the sorted F<sup>2+</sup> cells for self-renewal, primary spheres formed in F<sup>2+</sup> culture were individually picked up and transferred to separate wells, then dissociated into single cells. These single sphere-derived cells were then cultured for 7 days and assessed for secondary sphere formation. Spheres showing EGFP fluorescence similar to the primary spheres formed in almost all cases (59/60 wells). Such passage and formation of new

**TABLE 2**  
Characterization of Cells Fractionated by FACS Based on EGFP Fluorescence Intensity

	EGFP intensity		
	F-	F+	F++
(A) Immunocytochemistry			
%Nestin <sup>+</sup>	24.9 ± 14.4	68.0 ± 15.7	98.2 ± 0.3
%βIII-tubulin <sup>+</sup>	69.8 ± 12.0	37.5 ± 7.7	0.9 ± 0.3
(B) Labeling of cells with BrdU			
<i>In vivo</i>	3/262 (1.1%)	25/251 (10.0%)	68/251 (27.1%)
<i>In vitro</i>	9/289 (3.1%)	19/292 (6.5%)	58/172 (33.7%)
(C) Monolayer culture at low cell density			
Number of independent cultures	10	11	10
Plating efficiency (%)	2.8 ± 2.0	4.2 ± 3.0	11.0 ± 8.7
Frequency of clone types (%)			
N	52/71 (73.2)	77/135 (57.0)	30/221 (13.6)
A	8/71 (11.3)	18/135 (13.3)	16/221 (7.2)
N + A	11/71 (15.5)	40/135 (29.6)	175/221 (79.2)
Frequency of the cells that survived to form N + A clones (%)	0.4	1.2	8.7
Ratio	1	2.9	20

Note. FACS-sorted E14–15 cerebral cells (see Fig. 5) were subjected to immunocytochemical analysis (A), BrdU analysis (B), and low-density monolayer culture (C). (A) Cells gated into three fractions were plated onto PEI-coated coverslips, fixed immediately after they were attached, and subjected to immunocytochemical analysis. More than 300 cells were counted in each sample. (means ± SD, 3 experiments). (B) *In vivo*: E14 embryos whose mothers had been given BrdU twice (30 min apart) were removed 10 min after the second injection. Forebrain cells from the embryos were fractionated by FACS and immunostained immediately after the cells were attached to coverslips. The data presented are from one representative experiment and similar results were obtained in separate experiments with modified fractionation protocols. *In vitro*: FACS-sorted E15 forebrain cells were plated onto coverslips and cultured 12 h in DMEM/F12-based growth medium with EGF and bFGF (both 20 ng/ml) and then treated with BrdU (10 μM, 1 h) and immunostained with anti-BrdU. Data from one representative experiment are presented. Similar results were obtained in separate experiments with modified fractionation protocols. (C) E14 cerebral cells were plated onto PEI-coated plastic dishes at a low density (150 cells/0.2 cm<sup>2</sup> or 300 cells/0.4 cm<sup>2</sup>) and cultured for 7 days and then fixed and stained with cell-type-specific antibodies: βIII-tubulin for neurons, and GFAP for astrocytes. The frequencies of observation of clones consisting of neurons only (N), astrocytes only (A), and neurons plus astrocytes (N + A) are presented as the percentages of total clones obtained in 10 or 11 cultures (two independent experiments). Plating efficiencies are presented as the mean ± SD from the indicated number of cultures. The frequency of cells that survived to form N + A clones per total number of cells plated was obtained by [plating efficiency] × [frequency of N + A clones].

spheres was reproduced in several independent sets of experiments, and could be repeated for more than 3 months. The capacity to give rise to neurons, astrocytes, and oligodendrocytes was also confirmed by differentiation assays using (1) F<sup>2+</sup> cell-derived spheres passaged five or six times during 1–2 months (13 of 28 spheres), and (2) spheres formed by cells that were dissociated from spheres that had been passaged seven times and then—3 months after harvesting the original cells—sorted again into F<sup>2+</sup> (5 of 5 spheres). These results further indicated that the original cells gated into F<sup>2+</sup> could be maintained over an extended period of time, while exhibiting both multipotency and self-renewability.

#### E/nestin:EGFP Expression in the Adult CNS

We next examined the EGFP expression in the adult CNS of the E/nestin:EGFP-transgenic mice. By confocal

microscopic observation, EGFP fluorescence was seen along the lateral ventricle (Figs. 7B–7I) and its entire caudal extension to the spinal cord (Fig. 7J). In the cerebral periventricular area, a high level of EGFP (EGFP<sup>2+</sup>) was seen in a subset of ciliated ependymal cells, although some ependymal cells were only moderately fluorescent (EGFP<sup>+</sup>) (Fig. 7B). A moderate level of EGFP was also seen in the subependyma, which was S100 negative (Fig. 7C). Such EGFP<sup>+</sup> cells in the subependyma were Musashi1<sup>+</sup> (Figs. 7D–7F). This expression pattern was apparent as early as postnatal day (P) 2 (Fig. 7A).

As adult NSCs are known to be relatively quiescent and proliferate very slowly *in vivo* (Morshead et al., 1994), we also performed BrdU-labeling using two methods; one to detect cells proliferating very slowly (Doetsch et al., 1999) and another to detect proliferative cells for which the cell cycle length is very short (Morshead et al., 1994). By the former method (14-day label-

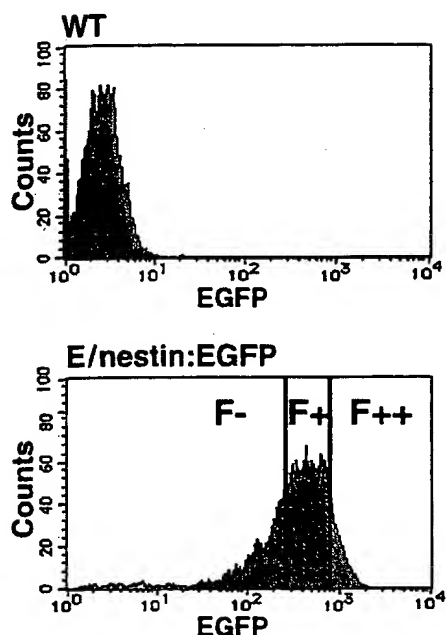


FIG. 5. Sorting of embryonic EGFP-expressing cells. FACS profiles of forebrain cells from E14 wild-type (WT) and E/nestin:EGFP mice. Anterodorsolateral cerebral fragments containing the "rostral focus" (Smart, 1983) were isolated from E14 transgenic mice. Dissociated cells were divided into  $F^-$  (30%),  $F^+$  (60%), and  $F^{2+}$  (10%) fractions by FACS. Note that there are no breaks between the three fractions on the histogram.

ing period followed by 7-day wash out), cells in the subependyma showing a moderate level of EGFP were BrdU<sup>+</sup> (Figs. 7G and 7H). By the latter method (6-h labeling period), BrdU<sup>+</sup> cells were again detected in the subependymal zone. However, these cells, which presumably corresponded to the transiently amplifying cells (non-NSCs) (Morshead *et al.*, 1994), were negative for EGFP (Fig. 7I). No BrdU<sup>+</sup> cells were detectable in the ependyma by either labeling method.

These results strongly suggest that NSCs, or at least regions in which NSCs exist, can be identified based on EGFP expression in E/nestin:EGFP-transgenic mice.

#### Adult EGFP<sup>+</sup> Cells become More Fluorescent in Culture and Form "Bright" Spheres

We next examined the relationship between E/nestin:EGFP expression in adult forebrain cells and the ability of these cells to generate neurospheres in suspension culture. Cells were harvested from the forebrain periventricular area (Fig. 8A) of adult nestin-

EGFP mice (three independent experiments using a total of 18 mice). EGFP-positive cells (both EGFP<sup>+</sup> and EGFP<sup>2+</sup>) were almost always (>90%) Musashi<sup>+</sup> and rarely GFAP<sup>+</sup> (<5%). EGFP<sup>2+</sup> cells were typically (>95%) S100<sup>+</sup>. Some of the S100<sup>+</sup> cells ( $51.4 \pm 10.7\%$ , mean  $\pm$  SD) and GFAP<sup>+</sup> cells ( $41.0 \pm 7.4\%$ ) were EGFP<sup>+</sup>. RC2 immunoreactivity was not detected, consistent with a previous report (Misson *et al.*, 1988) (not shown). Cells were sorted into the three ( $F^-$ ,  $F^+$ , and  $F^{2+}$ ) fractions by FACS (Fig. 8B), and cultured in the presence of EGF and bFGF.  $F^{2+}$  contained both EGFP<sup>2+</sup> and EGFP<sup>+</sup> cells. In  $F^{2+}$  cultures, brightly fluorescent spheres were formed (Figs. 8C–8E). Fluorescent spheres were also formed in cultures of  $F^+$  cells (Figs. 8F–8H), and in such cases, the  $F^+$  cells that were originally EGFP<sup>+</sup> became more fluorescent (usually within 24 h) and were EGFP<sup>2+</sup> at 4 days (Fig. 8G). The frequency of sphere formation was significantly higher in  $F^+$  ( $0.31 \pm 0.19\%$ ,  $n = 6$  wells) than  $F^{2+}$  ( $0.07 \pm 0.10\%$ ,  $n = 8$  wells) (Mean  $\pm$  SD,  $P < 0.05$  by Mann-Whitney's  $U$  test). In contrast, in  $F^-$  cultures, spheres were never generated and no intensification in fluorescence was observed (not shown). The adult-derived spheres, independent of their source ( $F^+$  or  $F^{2+}$ ), generated neurons, astrocytes,

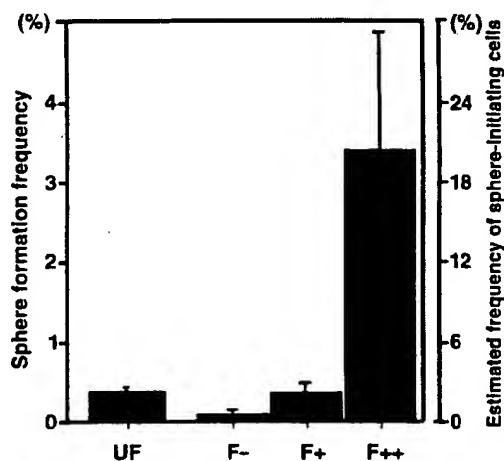


FIG. 6. Enrichment of sphere-initiating cells in the  $F^{2+}$  fraction. Fractionated E14 cerebral cells were cultured (10 cells/ $\mu$ l) in the presence of EGF and bFGF (both 20 ng/ml). The frequencies of sphere formation (>50  $\mu$ m in diameter) per total number of cells plated were obtained at 7 days. Data represent the mean  $\pm$  SD of 12 wells from two independent experiments. As controls, E14 cerebral cells that were applied to the cell sorter but not gated (unfractionated, UF) were cultured under the same conditions ( $n = 4$  wells). The estimated frequency of sphere-initiating cells (right scale) was obtained with the assumption that FACS-mediated cell damage was independent of EGFP fluorescence intensity (see text).

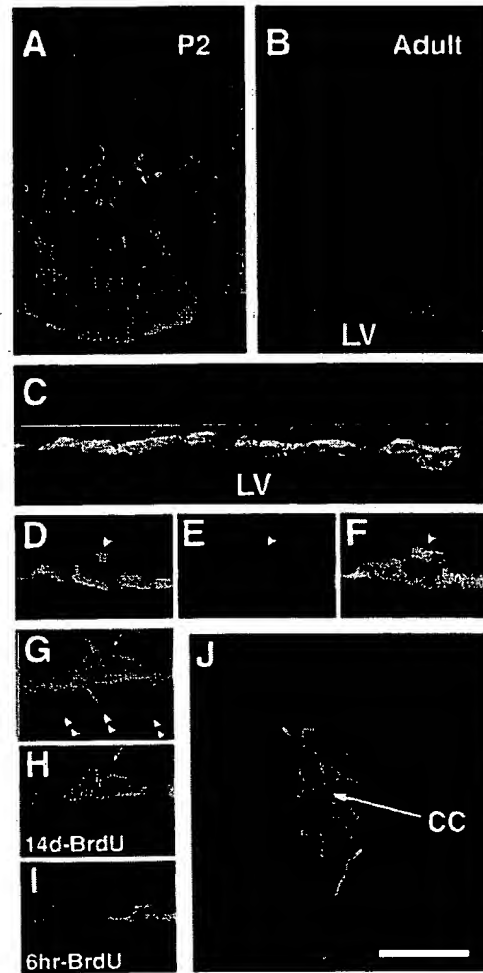
and oligodendrocytes when transferred onto PEI-coated dishes (7/11), and generated secondary spheres when dissociated and cultured further (5/5 for primary spheres; 5/5 for secondary spheres), just as the embryonic spheres had done. More than 95% of the cells comprising these forebrain-derived spheres were Nestin<sup>+</sup>, but were negative for GFAP and S100. Interestingly, of these Nestin<sup>+</sup> cells, 50–60% were strongly EGFP-positive and also RC2<sup>+</sup> (not shown). These *in vitro* cell properties were indistinguishable from those of embryonic NSCs. Thus, neurosphere propagation yielded the reemergence from adult tissue of a progenitor phenotype more typically associated with fetal brain development.

## DISCUSSION

By generating E/nestin:EGFP-transgenic mice and examining the developmental properties of FACS-fractionated cells, we demonstrated a direct correlation between the level of E/nestin-driven EGFP-expression visualized in cultured cells and their ability to proliferate, self-renew, and generate both neurons and glia. Moreover, our results also suggested that different developmental properties among undifferentiated cells could be inferred in live cells based on the level of EGFP expression.

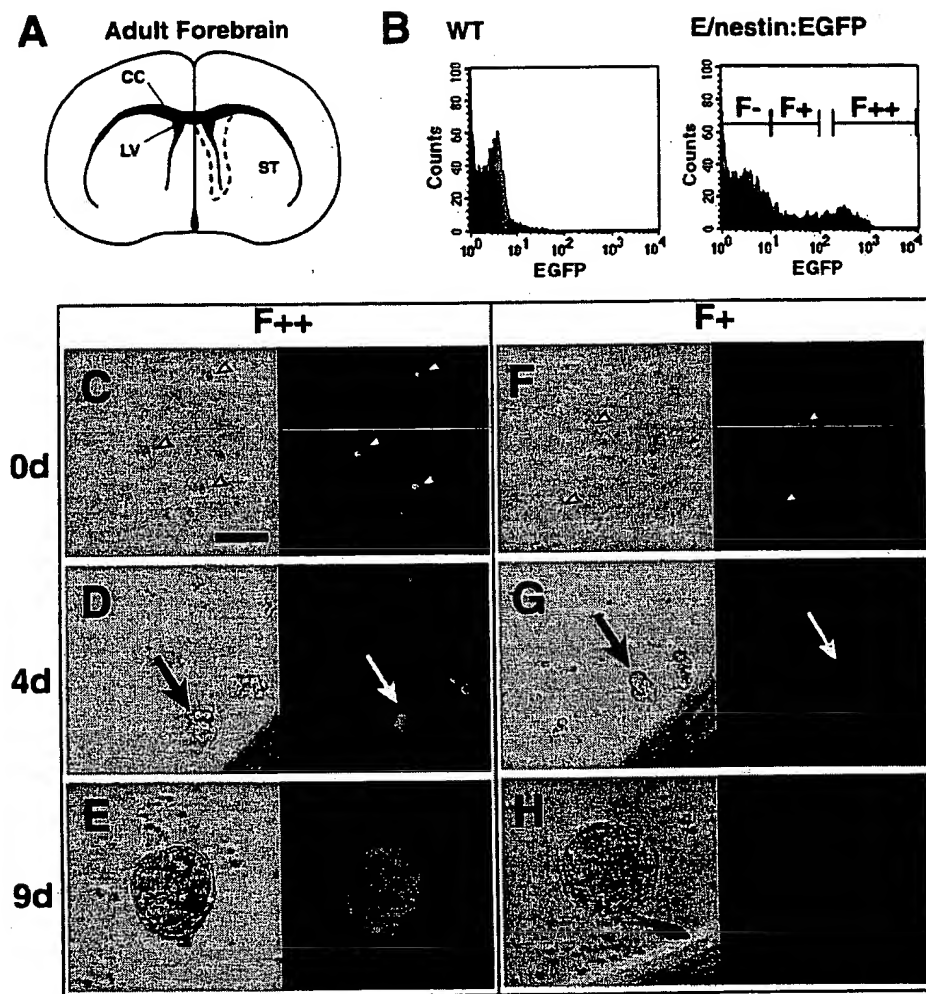
Our data also suggest that the *nestin* enhancer-driven EGFP intensity is an effective but nonexclusive marker for NSCs in the developing central nervous system. As shown in Fig. 5, histograms generated by the sorting of embryonic cerebral cells always showed a single peak with gradual tapering to both sides. This pattern suggests that the distribution of cells belonging to a given cell group may not be concentrated at a particular point (or small area) on the x-axis (EGFP intensity) of the FACS histogram. Other cell groups may be somewhat broadly distributed in a similar way. Thus, even within a cell population that had been categorized in our judgement as EGFP<sup>2+</sup>, the level of EGFP expression may not be uniform. This may also be the case for F<sup>-</sup>, F<sup>+</sup>, and F<sup>2+</sup>. This might be explained in part by the accumulation and stability of the EGFP, and by possible stochastic fluctuation of the transcriptional activation of the *nestin* second intronic enhancer. Although EGFP is advantageous for visualizing the properties of live cells, for the further purification of NSCs or lineage-restricted progenitor cells, it might be useful to sort cells by FACS using EGFP in combination with other cell surface markers.

We also cannot exclude the possibility that EGFP-



**FIG. 7.** EGFP expression in the postnatal and adult CNS. (A) Striatum at P2. EGFP expression was seen in the periventricular area, which contained EGFP<sup>+</sup> ependymal or process-bearing, tanycyte-like cells and EGFP<sup>+</sup> subependymal cells. (B) Adult striatum. EGFP fluorescence was seen in a segmental-like pattern, with EGFP<sup>2+</sup> cells in the ependyma and EGFP<sup>+</sup> cells in both the ependyma and the subependyma. LV, lateral ventricle. (C) Merged image of anti-S100 immunostaining (red) and EGFP fluorescence (green) in the adult periventricular area. S100-negative EGFP<sup>+</sup> cells were seen in the subependyma. (D–F) A magnified view, showing subependymal EGFP<sup>+</sup> cells (D) (arrowhead) were Musashi1<sup>+</sup> (red). E, F, a merged view of the data in D and E. (G) A cell in the adjacent subependymal zone (arrow) also expressed EGFP, but at a lower level (EGFP<sup>+</sup>). Double-arrowheads indicate cilia. (H) An EGFP<sup>+</sup> subependymal cell (arrow) that had incorporated BrdU that was administered for 14 days, followed by a 7-day washout period, to label slowly proliferating cells (Doetsch et al., 1999). (I) Short BrdU administration (6 h, 4 injections with 2-h intervals) did not label cells showing detectable levels of EGFP. Red, BrdU<sup>+</sup> nuclei. (J) In the spinal cord, EGFP fluorescence was confined to the ependyma encircling the central canal (CC). Among ependymal cells, however, the level of EGFP expression was not homogeneous, similar to the case in the forebrain. Bar: 40  $\mu$ m in A and B; 35  $\mu$ m in C and J; 30  $\mu$ m in D–I.





**FIG. 8.** Sorting of adult EGFP-expressing cells and sphere formation assay. (A) Schematic diagram of a frontal section of adult forebrain showing the dissected periventricular area (red broken lines). CC, corpus callosum. ST, striatum. LV, lateral ventricle. (B) FACS profiles of cells harvested from the forebrain periventricular area of wild-type (left) and E/nestin:EGFP (right) adult mice. Transgenic cells were sorted into three fractions ( $F^-$ ,  $F^+$ , and  $F^{++}$ ). Note the gap between  $F^+$  and  $F^{++}$ . (C-E) Sphere formation by sorted  $F^{++}$  cells. The indicated cells (arrowheads) at 0 day (C) showed an extremely high level of EGFP (EGFP $^{++}$ ), whereas the remaining cells were moderately fluorescent (EGFP $^+$ ). By 4 days (D), small fluorescent cell aggregates (arrow) had formed. By 9 days (E), fluorescent spheres had formed. These spheres generated neurons, astrocytes, and oligodendrocytes on PEI-coated coverslips (see text). (F-H) Intensified fluorescence and sphere formation in  $F^+$  cultures. Initially, the level of EGFP expression in  $F^+$  cells was "EGFP $^+$ " (arrowheads in F) or lower. However, cells with more intense fluorescence appeared during culture (usually within 24 h) and their level of EGFP expression was almost equivalent to that of the EGFP $^{++}$  cells in the  $F^{++}$  culture at 4 days (G). Cell divisions had occurred by this point, and small aggregates were found (arrow). By 9 days (H), spheres as brightly fluorescent as those in the  $F^{++}$  culture had formed. These spheres exhibited a similar differentiation potential as spheres formed in the  $F^{++}$  culture. In the  $F^-$  culture, spheres were never generated and no intensification of fluorescence was observed (not shown). Bar: 50  $\mu$ m in C-H.

negative multipotent progenitor cells exist in the embryonic brain, because spheres also formed in cultures of  $F^-$  cells ( $0.08 \pm 0.08\%$ ) (Fig. 6). However, compared with the frequency of sphere-initiating cells in  $F^{++}$  cells ( $F^-:F^{++} = 1:42.1$ ), such EGFP-negative stem cells are rare, even if they do exist.

In the adult E/nestin:EGFP-transgenic mice, it appears that the population of E/nestin:EGFP-defined cells include slowly dividing multipotent progenitor cells, which may be viewed as putative adult stem cells. This conclusion is based on the following results: (1) EGFP expression was confined to the periventricular

region, the likely reservoir of adult neural stem and progenitor cells (Luskin, 1988; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Pincus et al., 1998), (2) EGFP-expressing cells harvested from the periventricular regions of the E/nestin:EGFP-transgenic mice displayed neurosphere-forming activity (Reynolds and Weiss, 1992, 1996), while EGFP-negative cells did not, and (3) slowly dividing cells, which may be candidates for NSCs, showed a moderate level of EGFP expression, while rapidly dividing cells in the subependyma (non-NSCs) (Morshead et al., 1994) were negative for EGFP expression. As regards the origin of NSCs in the adult forebrain, Johansson et al. (1999) found that ependymal cells exhibit the cellular properties of stem cells. Doetsch (1999) and Chiasson et al. (1999) then used different methodologies to conclude that at least some subependymal cells may be adult NSCs. Here we showed that a subset of both ependymal and subependymal cells expressed EGFP fluorescence at heterogeneous intensities in E/nestin:EGFP-transgenic mice. Although our *in vitro* examinations of adult forebrain periventricular cells showed that sphere formation occurred more frequently in F<sup>+</sup> than in F<sup>2+</sup>, the heterogeneous EGFP expression pattern in the ependyma and subependyma prohibited us from determining which cell types in this area (ependymal cells or subependymal cells) predominated in the formation of the bright spheres. Further studies addressing the potential heterogeneity of E/nestin:EGFP-positive cells in the adult CNS using different approaches (e.g., Chiasson et al., 1999; Doetsch et al., 1999) are needed.

A particularly interesting finding was that moderately EGFP-expressing cells that were sorted into the F<sup>+</sup> fraction gained EGFP intensity during culture (within 24 h) (Fig. 8G). EGFP<sup>2+</sup> cells were highly enriched in the spheres derived from the adult CNS. These *in vitro* data are consistent with the previous *in vivo* findings that EGF infusion into the adult mouse forebrain increases the total amount and spatial distribution of Nestin staining (Craig et al., 1996). Thus, *nestin* enhancer in the adult NSCs is likely to be activated in the presence of EGF and bFGF *in vivo* and *in vitro*. Taken together with the developmental changes in the correlation between EGFP intensity and the frequency of sphere formation, these results suggest that adult CNS stem cells exist in a different microenvironment *in vivo* from that of the embryonic CNS stem cells, with respect to the presence of growth factors. Such environmental differences might be one reason for the different proliferative kinetics of the adult and embryonic CNS stem cells *in vivo* (Morshead et al., 1994; Martens et al., 2000).

In summary, by combining FACS and functional

analyses, we have characterized the EGFP-expressing cells of transgenic mice expressing EGFP under the regulatory control of the *nestin* enhancer. We have also obtained enriched populations of cells that are multipotent and self-renewing, definitive criteria for NSCs, from the developing murine CNS. Thus, this mouse line provides a useful tool for future efforts to dissect the molecular mechanisms regulating both the self-maintenance and phenotypic diversification of these cells *in vitro* and to analyze the biology of NSCs in their *in vivo* environment.

## EXPERIMENTAL METHODS

### Transgenic Mice

An evolutionarily conserved 637-bp (1162–1798) second intronic region of the rat *nestin* gene (Lothian and Lendahl, 1997) (from the Xh5 plasmid) was subcloned into the *Sma*I site of the hsp68-EGFP plasmid containing a 1.0-kb *Nru*I–*Bam*HI fragment of the minimum promoter of heat shock protein 68 (hsp68) (from the Ass-hsp68-*lacZ*-pA vector) and a 1.0-kb *Nru*I–*Bam*HI fragment containing the EGFP coding region and a polyadenylation signal from the pEGFP-N3 (Clontech Laboratories). The 2.7-kb *nestin*-hsp-EGFP fragment (*Sa*II) was purified, then injected into the pronucleus of fertilized mouse eggs. The minimum promoter of heat shock protein 68 (hsp68) exhibits no basal activity unless an enhancer is placed in its vicinity (Rossant et al., 1991). The base numbering of the rat *nestin* gene coincides exactly with the GeneBank submission, Accession No. AF004334. Six independent lines showing a similar expression pattern were established in a C57BL/6 background. The data presented were obtained from experiments using heterozygous mice from a single strain (#25). All procedures were performed in accordance with institutional guidelines. For timed pregnant mice, the date the vaginal plug was observed was defined as day 0.

### Cell Preparation and Sorting

Brain tissues were dissected from transgenic or wild-type mouse embryos in DMEM/F12 (GIBCO), then digested in 0.25% trypsin (GIBCO) at 37°C for 5 min. After quenching the digestion with medium containing ovomucoid inhibitor (0.7 mg/ml, Sigma), the material was triturated with a fire-polished Pasteur pipette. Fragments of the adult forebrain periventricular area were enzymatically digested as described previously

(Reynolds and Weiss, 1992). Dissociated cells were suspended in PBS containing 10  $\mu\text{g}/\text{ml}$  propidium iodide (PI), and filtered (30  $\mu\text{m}$ ). Cell sorting and analyses were performed using a FACS Vantage flow cytometer/cell sorter equipped with CELLQuest software (Becton-Dickinson). Cells ( $1\text{--}2 \times 10^6/\text{ml}$ ) were analyzed for forward scatter, side scatter, PI fluorescence, and EGFP fluorescence with an argon laser (488 nm, 100 mW) (Wang *et al.*, 1998). Dead cells were excluded by gating on forward and side scatter, and by eliminating propidium iodide-positive events. Viable cells from the transgenic mice were sorted into DMEM/F-12 medium at a speed of 3000 events/s. Sorted cells were kept on ice until used for further experiments.

### Cell Culture

For sphere cultures, sorted or unsorted cells were washed twice with DMEM/F12 and resuspended in DMEM/F-12-based serum-free growth medium containing insulin (25  $\mu\text{g}/\text{ml}$ ), transferrin (100  $\mu\text{g}/\text{ml}$ ), progesterone (20 nM), sodium selenate (30 nM), putrescine (60  $\mu\text{M}$ ), EGF (20 ng/ml), and bFGF (20 ng/ml) (all from Sigma) (Reynolds and Weiss, 1996). A 1:1 cocktail of this cell suspension and neurosphere conditioned medium was plated into each well at 10 cells/ $\mu\text{l}$ , lower than the cell density at which virtually all spheres were clonal (Hulpas *et al.*, 1997). Spheres that formed by 7–10 days *in vitro* were plated onto polyethylenimine (PEI)-coated coverslips and cultured further for 7–10 days in DMEM/F12 containing 1% FBS and  $10^{-6}$  M 13-cis-retinoic acid (Nakarai, Japan). Seven-day-old spheres were mechanically dissociated into single cells, and the cells were cultured further for sphere formation.

For monolayer cultures, the culture surface of PEI-coated plastic dishes was divided into separate zones by means of three silicone rubber rings (5–7 mm inner diameter, 1 mm thick), outside of which astrocytes from perinatal wild-type mice were seeded as a feeder cell layer (Miyata and Ogawa, 1994). When astrocytes had formed a confluent monolayer, sorted cells ( $F^-$ ,  $F^+$ , and  $F^{2+}$ ) or unsorted cells suspended in the DMEM/F12-based growth medium supplemented with 2% FBS were individually plated onto the three circular areas (coated with 10  $\mu\text{g}/\text{ml}$  fibronectin, Sigma) at a density of 750 cells/ $\text{cm}^2$ . Just before plating, single cell dissociation (>98%) was confirmed under an inverted microscope (20 $\times$ ). After cells attached to the dishes (within 1 h), the silicon rings were removed, and fields containing clusters of >1 cell were excluded from analysis. Sorted cells were damaged more often than unsorted

cells even though all cells had been viable at the time they were plated. Since most cell death occurred within 24 h in the low-density cultures of the sorted cells, the actual cell density on the next day was estimated to be <100 cells/ $\text{cm}^2$  for the sorted cells. The possibility of feeder cells contaminating the fields of interest was excluded by daily observation. Half the medium was renewed every 2–3 days, and cells were fixed on day 7 or in some cases on days 1 and 3 (unsorted cells). Cells were observed under an inverted fluorescent microscope (IX70, Olympus, Japan) equipped with a cooled CCD digital camera (SPOT2, Seki Technotron, Japan), using an FITC filter set. Despite the reported stability of EGFP in mammalian cells, we observed a rapid disappearance of E/nestin:EGFP when cells were allowed to differentiate (within 12–24 h) (Figs. 3A, 3B, and 4B).

### Immunocytochemistry

Frozen sections (12  $\mu\text{m}$ ) of brains fixed with 4% paraformaldehyde were prepared as described previously (Sakakibara *et al.*, 1995). Unsorted or sorted cells were attached to PEI-coated coverslips, then fixed in 4% paraformaldehyde. Cellular sheets formed by spheres were fixed similarly. Cells were then stained with mouse anti-Nestin (Rat401, Hybridoma Bank), anti- $\beta$ III-tubulin (Sigma), anti-MAP2 (Sigma), anti-GFAP (rabbit, DAKO or mouse IgG, Sigma), O4 (mouse IgM, Boehringer-Mannheim), anti-Mushashi1 (rat IgG), RC2 (mouse IgM, from Dr. Yamamoto, Tsukuba Univ., Japan), anti-S100 (rabbit, MEDAC), anti-PSA-NCAM (mouse IgM, from Dr. Seki, University of Juntendo, Japan), and anti-GFP (rabbit, Clontech), as described (Kaneko *et al.*, 2000). EGFP was readily detected in fixed specimens but was more bleached than in live cells. Anti-GFP did not show nonspecific staining, and was used for double or triple staining. Live cells that were judged as EGFP<sup>2+</sup> were almost always (98–100%) immunocytochemically EGFP<sup>2+</sup>. Specimens were examined using a confocal microscope (LSM510, Carl Zeiss) or a universal fluorescent microscope (Axiophoto 2, Carl Zeiss). Digital images were captured with a CCD camera (FUJIFILM HC-200 equipped with Photograb II software).

### BrdU Labeling

FACS-sorted cells were suspended in medium containing EGF and bFGF, plated onto PEI-coated coverslips ( $1 \times 10^5$  cells/ $\text{cm}^2$ ), and incubated overnight. They were then treated with BrdU (10  $\mu\text{M}$ ) for 1 h, fixed in 4% paraformaldehyde, and immunostained with anti-

GFP first, followed by FITC-conjugated secondary antibody. Cells were then refixed with the same fixative, pretreated with 2 N HCl containing 0.01% Triton X-100, and incubated with anti-BrdU (mouse IgG, 1:200; Sigma), followed by rhodamine-conjugated secondary antibody.

For labeling *in vivo*, timed pregnant female mice bearing nestin-EGFP transgenic embryos were injected twice with BrdU (0.1 mg/g body weight of the pregnant mouse for each injection, 30 min apart) and the embryos were isolated 10 min after the second injection. Forebrain cells were harvested from the BrdU-treated embryos, sorted by FACS, then plated onto coverslips. One hour later, the cells were fixed and immunostained with anti-BrdU. To label slowly dividing cells in the adult brain, BrdU (1 mg/ml) was given to mice in their drinking water for 2 weeks, and the cells were harvested 1 week later. To label rapidly dividing cells in the adult brain, BrdU was administered four times by intraperitoneal injection (0.1 mg/g body weight, 2 h apart, 6 h total labeling time).

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# RNA-binding protein Musashi family: Roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation

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Homologues of the Musashi family of RNA-binding proteins are evolutionarily conserved across species. In mammals, two members of this family, Musashi1 (Msi1) and Musashi2 (Msi2), are strongly coexpressed in neural precursor cells, including CNS stem cells. To address the *in vivo* roles of *msi* in neural development, we generated mice with a targeted disruption of the gene encoding Msi1. Homozygous newborn mice frequently developed obstructive hydrocephalus with aberrant proliferation of ependymal cells in a restricted area surrounding the Sylvius aqueduct. These observations indicate a vital role for *msi1* in the normal development of this subpopulation of ependymal cells, which has been speculated to be a source of postnatal CNS stem cells. On the other hand, histological examination and an *in vitro* neurosphere assay showed that neither the embryonic CNS development nor the self-renewal activity of CNS stem cells in embryonic forebrains appeared to be affected by the disruption of *msi1*, but the diversity of the cell types produced by the stem cells was moderately reduced by the *msi1* deficiency. Therefore, we performed antisense ablation experiments to target both *msi1* and *msi2* in embryonic neural precursor cells. Administration of the antisense peptide-nucleotides, which were designed to specifically down-regulate *msi2* expression, to *msi1*<sup>-/-</sup> CNS stem cell cultures drastically suppressed the formation of neurospheres in a dose-dependent manner. Antisense-treated *msi1*<sup>-/-</sup> CNS stem cells showed a reduced proliferative activity. These data suggest that *msi1* and *msi2* are cooperatively involved in the proliferation and maintenance of CNS stem cell populations.

**D**uring mammalian CNS development, neurons and glial cells are thought to be generated from common neural precursor cells (CNS stem cells) located in the periventricular area (1). The molecular basis for the maintenance of this cell population is, however, largely unknown. The recent discovery of neural RNA-binding proteins raises the possibility that the development of neural cells from their precursors may be controlled by posttranscriptional gene regulation, including mRNA stabilization, splicing, or translational control. Musashi1 (Msi1) and Musashi2 (Msi2) are RNA-binding proteins that are characterized by two RNP-type RNA recognition motifs (RRMs) and show remarkable similarity to one another, both in their primary structures and their RNA-binding specificities *in vitro* (2–4). These two molecules seem to define the Msi family of RNA-binding proteins, which is evolutionarily conserved across different species. In mammals, Msi1 and Msi2 expression is developmentally regulated. Our previous studies revealed that Msi1 and Msi2 are coexpressed predominantly in proliferating embryonic pluripotent neural precursors (2, 4–6), as well as in cell

populations that are believed to be the source of postnatal and adult CNS stem cells (3, 6). In the cerebral cortex, the expression of Msi1 and Msi2 is rapidly down-regulated in newly generated postmitotic neurons (2), with the exception of some GABAergic interneurons that continue to express Msi2 exclusively (4). Although the molecular functions of the Msi family members remain obscure, their expression profiles suggest that they may play similar roles in the development and maintenance of CNS stem cells through posttranscriptional gene regulation (6).

In the present study, we used targeted disruption of the *msi1* gene in mice and antisense ablation of Msi2 to address the roles of these proteins during development. The results suggested that the Msi family genes have critical functions in restricted cell populations, including CNS stem cells.

## Materials and Methods

**Gene Targeting.** Genomic DNA fragments of *msi1* gene (1.5-kb *NotI*–*SalI* fragment for the left arm, and 9.4-kb *HindIII*–*SalI* fragment for the right arm) were isolated from 129/Sv genomic DNA library and inserted into the targeting vector, which contained a 1.8-kb G418 resistance (*neo*) cassette and the diphtheria toxin A-fragment (DT-A) cassette. This targeting construct was designed to delete 4.2 kb of genomic DNA, including a 267-bp exonic sequence spanning the translational initiation codon and the first RRM, and to replace them with the *neo* cassette in the same transcriptional orientation as *msi1*. Correctly targeted ES cells, chimeric males, and the progeny of heterozygous intercrosses were genotyped by Southern blot or PCR analysis, using WT and mutant allele specific primers (primer sequences available on request). After seven to eight generations of backcrossing heterozygous mutants and C57BL/6 females, the F<sub>8</sub> or F<sub>9</sub> progeny of heterozygous intercrosses was used for histological and *in vitro* culture analyses. Histological analyses were performed as described (2, 4).

**Antisense Peptide Nucleic Acids (asPNA).** The asPNAs were custom synthesized, purified, and analyzed by PE Biosystems. The sequences of the *msi2* asPNA corresponded to the translation initiation codon (PNA1, 5'-CTCCATAGCGGAGCC-3'-Lys) or the coding region (PNA2, 5'-ACCTAATACTTTATCT-3'-Lys).

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Abbreviations: Msi1, Musashi1; Msi2, Musashi2; VZ, ventricular zone; SVZ, subventricular zone; SCO, subcommissural organ; SFC, sphere-forming cell; RRM, RNA recognition motifs; asPNA, antisense peptide nucleic acids; CC, corpus callosum; CSF, cerebrospinal fluid; BrdU, 5-bromodeoxyuridine.

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These two asPNAs had similar effects on neurosphere formation, and PNA1 was used throughout the experiments in this report. A scrambled PNA (5'-AACTCTTATATCTCTA-3'-Lys), which did not match significantly any other known RNA or DNA sequence, was used for the control.

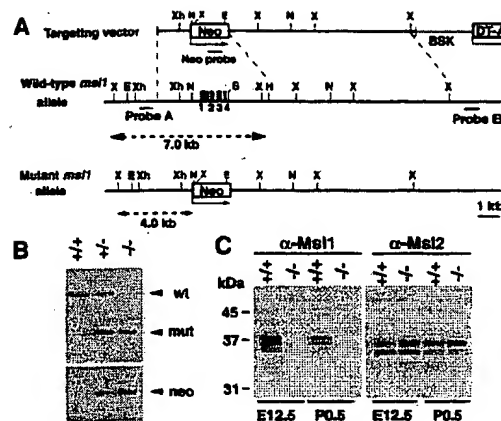
**Neurosphere Culture.** The basic culture medium, containing 20 ng/ml epidermal growth factor (Sigma) and 10 ng/ml FGF2 (R & D Systems), and the procedures for the neurosphere formation and differentiation assays were as described (7). Briefly, cells from the anterior halves of the E14.5 telencephalon were used for primary sphere formation ( $1 \times 10^5$  cells per ml). The indicated amount (0–10  $\mu$ M) of asPNA was added to the culture medium when the primary spheres were dissociated and replated for secondary sphere formation (500 cells per 200  $\mu$ l per well, 96-well plate). The numbers of secondary spheres were counted 4 days later (4 div). Cells used for semiquantitative RT-PCR and immunocytochemistry for Msi2 or nestin (Rat401) were harvested after 24 h (1 div) of treatment with the asPNA. To assess cell proliferation within the asPNA-treated spheres, 2  $\mu$ M BrdU (5-bromodeoxyuridine, Sigma) was administered to the cultures at 2 div. After 24 h of incubation, spheres were trypsinized, dissociated, and plated onto polyL-lysine-coated coverslips for 3 h, then processed for immunocytochemistry using anti-BrdU antibody (Sigma).

**Semiquantitative RT-PCR.** Cells incubated with or without asPNA (10  $\mu$ M) for 24 h were collected ( $1.5 \times 10^5$  cells). Semiquantitative RT-PCR was carried out using the primer sets for *msi2* (5'-GTCTGCGAACACAGTAGTGGAA-3' and 5'-GTAGCCTCTGCCATAGGTTGC-3'; 340 bp), *AUF1* (5'-ACTGCACTCTGAAGTTAGATCCTA-3' and 5'-TGTAGCTATTTTG-ATGTCCACCTC-3'; 534 bp), *hnrNP1* (5'-ATGGCTAGTGCTTCACTCCAGTCA-3' and 5'-CTGTGCTTGGCTGAGTTCACAAA-3'; 508 bp), *hnrNPC1/C2* (5'-AGTGGATTCAATTCGAAGAGTGGAA-3' and 5'-GATGACCTGGTGTACTTTCATCT-3'; 513 bp), *NKT* (5'-TAAGCTGAGTATAGAGGTGCT-3' and 5'-CATTCACTAGGATACAGATGC-3'; 332 bp), and *g3pdh* (5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'; 452 bp).

## Results

***msi1*<sup>-/-</sup> Mice Develop Obstructive Hydrocephalus with Ependymal Abnormalities.** Targeted disruption of the *msi1* locus in ES cells was performed by replacing four exons, including the translation initiation codon, with a neo-resistance gene cassette (Fig. 1A and B). Interbreeding of the heterozygous mutant (*msi1*<sup>+/-</sup>) mice yielded homozygous mutant (*msi1*<sup>-/-</sup>) pups with the expected Mendelian ratio, indicating that Msi1 is not essential for embryonic viability. The absence of Msi1 expression in embryonic and neonatal brains in homozygous animals was confirmed by immunoblot analysis (Fig. 1C) using an anti-Msi1 monoclonal antibody.

Histological analyses showed that *msi1*<sup>-/-</sup> embryos (E10–E17) exhibited normal cortical development. Neuroepithelial cell proliferation in the ventricular zone (VZ) was not perturbed, as determined by light microscopy and BrdU incorporation analysis (not shown). Newborn *msi1*<sup>-/-</sup> pups were normal in size and appearance for the first postnatal week. However, by 1–2 postnatal weeks, 70–80% of the *msi1*<sup>-/-</sup> animals developed hydrocephalus with progressive dilation of the lateral ventricles and an enlarged, domed cranium (Fig. 2E–I). In addition, a small population of *msi1*<sup>-/-</sup> mice (5–10%) exhibited Probst's bundle (8), an entangled axon tract in the corpus callosum (CC), because of errors in the projection of commissural fibers across the midline in the forebrain (Fig. 2A–D). This callosal agenesis was observed at E17, when normal CC axons have arrived at the midsagittal plane (not shown). Agenesis or hypoplasia of the CC



**Fig. 1.** Targeting strategy, germ-line transmission, and expression analysis of the *msi1* gene. (A) Organization of the targeting vector, the *msi1* gene, and the allele resulting from homologous recombination. Four exons (black boxes) of the *msi1* allele containing the initiation codon were replaced with a Neo cassette. A 0.3-kb *Sau3AI* fragment (probe A) was used to screen for recombinant alleles, and the sizes of the recombinant and WT fragments after *XbaI* digestion are shown (broken lines). X, *XbaI*; E, *EcoRV*; Xh, *XhoI*; Not, *NotI*; S, *SalI*; H, *HindIII*; BSK, plasmid vector. (B) Germ-line transmission was confirmed by Southern blot analysis of *XbaI*- or *EcoRV*-digested tail DNA from a litter of F<sub>2</sub> mice using probe A or the Neo probe, respectively. (C) Immunoblot analysis of brain lysates from E12.5 and P0.5 mice using anti-Msi1 or anti-Msi2 antibodies. Genotypes are indicated.

with Probst bundles is frequently found in hereditary hydrocephalus in humans (X-linked hydrocephalus) (9) and in several targeted and spontaneous mouse mutants, e.g., *hyh* (10) and *E2F-5* (11), suggesting that complex multigenic developmental pathways are disrupted in the *msi1*<sup>-/-</sup> mice.

Pathologic examination of the *msi1*<sup>-/-</sup> adult brains revealed dilation of the lateral ventricles and the third ventricle, thinning of the cerebral cortices, hypoplasia of the septa, disruption of the white and gray matters accompanied by intracerebral hemorrhage, edema, and necrosis of the periventricular parenchyma. The fourth ventricle was not dilated in *msi1*<sup>-/-</sup> mice. The olfactory bulb, cerebellum, thalamus, medulla, and hippocampus were not primarily affected, although they appeared to be compressed by the elevated intracranial pressure (Fig. 2F and I). The dilation of the lateral ventricles was frequently accompanied by cavitation in the subventricular zone (SVZ) (Fig. 2O'). The areas surrounding the anterior horns of the lateral ventricles were occasionally denuded of ependymal cells or had a disrupted ependymal lining (Fig. 2O'). Analyses of P0 forebrain sections by BrdU labeling, TUNEL staining, and immunohistochemistry using cell-type-specific markers failed to demonstrate significant changes in the number of apoptotic cells, differentiated neurons, or proliferating precursor cells in the *msi1*<sup>-/-</sup> SVZ before the onset of ventricular dilation (not shown). The *msi1*<sup>-/-</sup> mice with severe hydrocephalus demonstrated ataxic gate and dehydration, and they eventually died within 1–2 months after birth. Moderate cases exhibited the dilated lateral ventricles and cavitation of the septum pellucidum (Fig. 2H) but survived into adulthood.

In the *msi1*<sup>-/-</sup> mice, abnormal proliferation and polyposis were observed in ependymal cells surrounding the Sylvius aqueduct, as well as in the subcommissural organ (SCO), an ependymal gland located at the entrance of the cerebral aqueduct that secretes glycoproteins into the cerebrospinal fluid (CSF) (12) (Fig. 2K and L). These glycoproteins are known to aggregate in the CSF to form a fibrous structure (Reissner's fiber) along the aqueduct (12). Notably, Msi1 is expressed in the ependymal cells of both the SCO and the cerebral aqueduct (Fig.

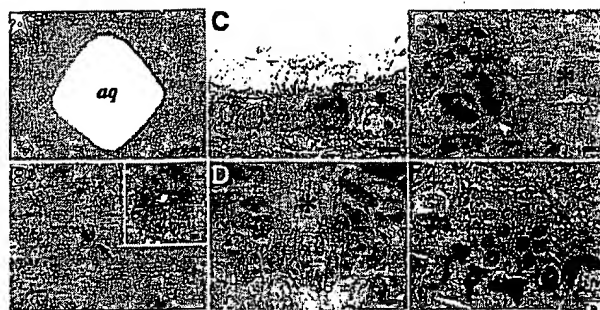






**Fig. 2.** Development of hydrocephalus in postnatal *msi1*<sup>-/-</sup> mice with ependymal abnormalities and the concurrent expression of *Msi1* and *Msi2* in the developing CNS and aqueduct. Genotypes are indicated. (A–I) Hematoxylin & eosin-stained adult brain. Agensis of the CC observed in an adult *msi1*<sup>-/-</sup> mouse (B, arrowheads) compared with the normal CC in a WT littermate (A). (C and D) A higher magnification view of the normal CC and the Probst's bundle, respectively. (E–I) Hydrocephalus of the adult *msi1*<sup>-/-</sup> mice. (E and F) Sagittal sections. (G–I) Coronal sections at the level of the anterior commissure. (F and I) A severe hydrocephalic mutant showing massive ventricular dilation. (H) Moderate dilation of the lateral ventricles accompanied by cavitation of the septum pellucidum and hypoplasia of the septum. (J–L) Coronal sections through the aqueduct of the P7 brain, showing abnormal accumulation and polyposis of ependymal cells surrounding the Sylvius aqueduct and SCO. (M and N) mRNA *in situ* hybridization analysis of *msi1* and *msi2* in the WT E12.5 telencephalon showing their expression in the VZ. (N and N') Immunohistochemical detection of *Msi1* and *Msi2* in ependymal cells surrounding the aqueduct of WT P3 mice. Immunoreactivities were visualized by DAB (brown), and the nuclei were counterstained with hematoxylin. (O and O') Expression of *Msi2* in the SVZ and ependymal cells lining the lateral ventricle of a hydrocephalic *msi1*<sup>-/-</sup> (O') and a WT littermate (O) at P3. The *msi1*<sup>-/-</sup> tissue shows partial destruction of the SVZ (arrow). *lv*, lateral ventricle; *aq*, Sylvius aqueduct; *+*, septum pellucidum. (Scale bars: A and B, 150  $\mu$ m; C, D, and J–L, 50  $\mu$ m; E–I, 1 mm; M, 30  $\mu$ m; N, 10  $\mu$ m; O, 100  $\mu$ m.)

2N). Electron microscopic analysis showed an abnormal stratification of the ependymal cells of the *msi1*<sup>-/-</sup> aqueduct that was not evident in heterozygous or WT littermates. Detailed histological analysis demonstrated that the aqueduct of *Msi1* null mice was enveloped by a thick epithelium composed of two or three layers of ependymal cells, in contrast to the well organized single ependymal cell layer in the aqueduct of WT or heterozygous littermates (Fig. 3D). Although the majority of these *msi1*<sup>-/-</sup> cells appeared to be terminally differentiated ependymal cells, characterized by cilia (Fig. 3D and F), some appeared to proliferate heterotopically without nuclear heteromorphism (Fig. 3E), which is consistent with the ependymal polyposis in the



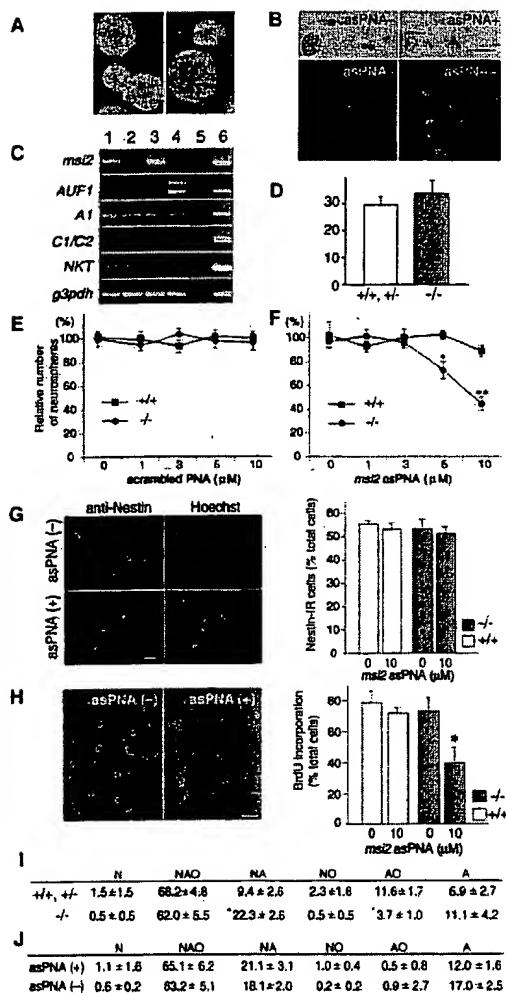
**Fig. 3.** Light microscopic and ultrastructural analyses of the *msi1*<sup>-/-</sup> ependyma at P14. (A and B) Light micrographs of the *msi1*<sup>-/-</sup> brain showing stenosis of the Sylvius aqueduct (B, arrow) and of the WT brain (A). *Inset* in B shows a low-power view of the ependymal cells in the aqueduct. (C) An electron micrograph of the WT aqueduct showing a single layer of ependymal cells and well organized microvilli protruding into the aqueductal lumen. (D) The aqueduct of an *msi1*<sup>-/-</sup> mouse, surrounded by the stratified ependymal epithelium composed of two or three cell layers. (E) A high-power view of *msi1*<sup>-/-</sup> ependymal cells, showing heterotopic mitotic figures without nuclear heteromorphism (arrowhead). Cytoplasmic organelles in these cells appear intact. The lumen of the *msi1*<sup>-/-</sup> aqueduct is completely filled with amorphous materials (asterisks in D and E). (F) A magnified view of *msi1*<sup>-/-</sup> ependymal evaginations and cilia. Cilia, characterized by central and peripheral tubules (arrows), are apposed to and are disarranged by solid materials (dashed line) that are abundant in the electron-dense particles of glycogen granules (arrowheads). (Scale bars: A and B, 40  $\mu$ m; B *Inset*, 8  $\mu$ m; C and D, 5  $\mu$ m; E, 2  $\mu$ m; F, 0.5  $\mu$ m.)

cerebral aqueduct and SCO. The lumen of the *msi1*<sup>-/-</sup> aqueduct was completely filled with amorphous materials abundant in electron-dense glycogen granules, and the microvilli of ependymal cells were irregularly arranged, as if they were compressed by the packed materials (Fig. 3F).

Hydrocephalus could result from impaired CSF absorption from the subarachnoid space or from overproduction of CSF by the choroid plexus; however, histological examination failed to demonstrate abnormalities in these areas (not shown). Taking these observations together, it is reasonable to attribute the hydrocephalus of *msi1*<sup>-/-</sup> mice to the obstruction of SCF flow in the aqueduct.

**Characterization of CNS Stem Cells Lacking *Msi1*.** We next examined the role of *Msi1* in the CNS stem cell population using a neurosphere assay, which is a selective culture system for CNS stem cells (7, 13). In the presence of mitogens such as epidermal growth factor and fibroblast growth factor 2, each CNS stem cell proliferates to form a floating multicellular structure, the neurosphere. Thus, CNS stem cells can be defined as sphere-forming cells (SFCs) (13). The number and size of the formed neurospheres are believed to reflect the number and proliferative activity of SFCs in the dissociated cell population, respectively (neurosphere formation assay). The multipotency of SFCs, that is, their ability to generate different neural cell types, can be assessed by a neurosphere differentiation assay; each neurosphere can be induced to differentiate into TuJ1<sup>+</sup> neurons (N), GFAP<sup>+</sup> astrocytes (A), and/or O4<sup>+</sup> oligodendrocytes (O), after being plated onto substrates in a culture medium containing serum. Consistent with the histological analysis of the *msi1*<sup>-/-</sup> embryos, the number and size of the neurospheres derived from the *msi1*<sup>-/-</sup> embryos were quite similar to those derived from WT littermate embryos (Fig. 4A and D). When the *msi1*<sup>-/-</sup> neurospheres were dissociated into single cells, a subset of the cells reformed neurospheres repeatedly, for as many passages as dissociated cells from the WT neurospheres did (not shown). These observations confirmed that the characteristics of the





**Fig. 4.** Loss of *msi1* and *msi2* activities decreased the efficiency of neurosphere formation through the reduced proliferation of SFCs. (A) Neurospheres derived from the E14.5 telencephalons of *msi1*<sup>-/-</sup> or WT littermates (after three passages). (Scale bar, 100  $\mu$ m.) (B) Suppression of Msi2 immunoreactivity on the asPNA-treated SFCs. Dissociated cells from the WT (Upper) or *msi1*<sup>-/-</sup> (Lower) primary neurospheres were incubated with or without asPNA (10  $\mu$ M) for 24 h and immunostained with anti-Msi2 antibody. Immunoreactivities to Msi2 were visualized by a DAB reaction (brown, Upper) or Alaxa-568 (red, Lower). Insets are magnified views of representative cells, showing the repression of Msi2 protein expression without pyknotic changes. [Scale bar, 5  $\mu$ m (Inset) or 25  $\mu$ m (Upper and Lower).] (C) Semiquantitative RT-PCR analysis of *msi2* mRNA, the *msi*-related genes encoding RNA-binding protein (*AUF1*, *hnRNP A1*, and *hnRNP C1/C2*), *NKT* mRNA, and an internal control mRNA (*g3pdh*). The *msi1*<sup>-/-</sup> or WT SFCs were incubated with or without asPNA for 24 h, then subjected to the RT-PCR. Lane 1, WT cells; lane 2, WT cells + asPNA; lane 3, *msi1*<sup>-/-</sup> cells; lane 4, *msi1*<sup>-/-</sup> cells + asPNA; lane 5, no RT control; lane 6, E14.5 WT cerebral cortex. (D) The number of neurospheres per field ( $\times 4$ ) formed by the dissociated cells from *msi1*<sup>-/-</sup> (-/-, *n* = 3) or littermates (+/+, +/-, *n* = 3). (E and F) The number of neurospheres derived from the telencephalons of *msi1*<sup>-/-</sup> and their WT littermates in the presence of the scrambled PNA (E) or in the presence of the asPNA (F). The data were presented as the mean  $\pm$  SEM (WT, *n* = 3–6; *msi1*<sup>-/-</sup>, *n* = 3–7). \**P* < 0.005, \*\**P* < 0.0001, in comparison with the WT control (unpaired *t* test). (G) The number of nestin<sup>+</sup> cells after the exposure to asPNA for 24 h. Dissociated cells of the primary neurospheres derived from the WT and *msi1*<sup>-/-</sup> telencephalons were incubated with or without asPNA (10  $\mu$ M) and then immunopositive cells for anti-nestin were counted (WT, *n* = 6; *msi1*<sup>-/-</sup>, *n* = 6). Photomicrographs showed the *msi1*<sup>-/-</sup> cells that were positive for nestin (green). (Scale bar, 25  $\mu$ m.) (H) Decreased number of BrdU<sup>+</sup> cells within the *msi1*<sup>-/-</sup> neurospheres

*msi1*<sup>-/-</sup> stem cells *per se* were unchanged; disruption of the *msi1* gene alone did not affect the number or self-renewal activity of CNS stem cells.

A clonogenic differentiation assay using cell-type-specific markers revealed that the majority of SFCs from *msi1*<sup>-/-</sup> mice were multipotent and could give rise to all three types of differentiated cells (NAO) (Fig. 4I). Nonetheless, we noticed that the *msi1*<sup>-/-</sup> SFCs showed a slightly limited repertoire for differentiation: the number of clones containing oligodendrocytes (AO+NO+NAO) tended to be slightly smaller in the *msi1*<sup>-/-</sup> neurospheres (66.2%) than in the WT neurospheres (82.1%). This reduction could not be restored by supplementing the differentiation medium with retinoic acid or T3, agents that potentiate the differentiation and proliferation of oligodendrocyte precursors (not shown). Thus, a lack of *msi1* activity reduces the multipotency of the CNS stem cells.

**The Msi Family Functions in the Proliferation of Neural Progenitor Cells Including CNS Stem Cells.** Taking into account the high expression levels of *msi1* during embryonic development (2), it was somewhat surprising that *msi1*<sup>-/-</sup> mice survived embryonic development with just minor structural abnormalities in restricted populations of ependymal cells. This finding indicates that *msi1* is not essential for the development of most tissues and body structures. In addition, a small fraction of *msi1*<sup>-/-</sup> mice (5–10%) survived into adulthood without detectable morphological abnormalities. These observations raised the possibility that the loss of *msi1* functions might be compensated for, at least in part, by one or more other genes. One such candidate was *msi2*, another member of the Msi family (4). In the CNS, the expression of Msi2 is developmentally regulated and overlaps with that of Msi1 in neural precursor cells, astrocytes, and ependymal cells (Fig. 2M–O) (4). The Msi2 expression level was up-regulated 1.4–2.0-fold in the *msi1*<sup>-/-</sup> embryonic brains (E12.5), compared with WT brains, as determined by Western (Fig. 1C) and Northern (not shown) blotting. These results support the hypothesis that *msi2* compensates for the *msi1* deficiency during the CNS development of *msi1*<sup>-/-</sup> mice.

To test directly for the cooperative involvement of Msi family proteins in the function of CNS stem cells, we tried making a functional double knockout of *msi1* and *msi2*. To this end, we added antisense compounds specific to the *msi2* gene to the medium of a CNS stem cell culture prepared from *msi1*<sup>-/-</sup> embryos or WT littermates and assessed the resulting number of neurospheres. Antisense oligonucleotides covering the translation initiation region or the coding region of *msi2* (a 16-mer) was synthesized as peptide nucleic acids (asPNA). PNA is a new type of DNA analogue that has an artificial homomorphous peptide backbone with much higher sequence specificity for target genes,

exposed to asPNA. During the formation of neurospheres in the presence (+) or absence (-) of asPNA (10  $\mu$ M), BrdU was administered at 2 div. After the additional cultivation for 24 h, each sphere was dissociated and immunostained with anti-BrdU (WT, *n* = 6; *msi1*<sup>-/-</sup>, *n* = 6). Photomicrographs represented the BrdU-labeled *msi1*<sup>-/-</sup> cells (green). The data were presented as the mean  $\pm$  SEM. \*, *P* < 0.001 in comparison with the other conditions (two-tailed Student's *t* test). (Scale bar, 10  $\mu$ m.) (I) Differentiation assay of neurospheres that derived from the *msi1*<sup>-/-</sup> (-/-) and their littermate cells (+/+, +/-). The differentiation capacity of each primary neurosphere was determined based on the cell types contained in each clone. N, neurons; A, astrocytes; O, oligodendrocytes. The majority (84.5%) of neurospheres from *msi1*<sup>-/-</sup> mice generated both neurons and glia (NAO+NA+NO). The clone types were analyzed for 98 spheres from WT or heterozygous mice (*n* = 5) and 234 spheres from *msi1*<sup>-/-</sup> mice (*n* = 10) and presented as the mean  $\pm$  SEM. \*, *P* < 0.0001, in comparison with WT and heterozygous controls (Student's *t* test). (J) Differentiation assay of neurospheres that derived from the *msi1*<sup>-/-</sup> cells treated with or without asPNA. The clone types were analyzed for 67 spheres from asPNA (+) and 141 spheres from asPNA (-).

a higher resistance to proteases and nucleases, and lower cytotoxicity (14) than other forms of antisense nucleotides, such as the phosphorothioate oligos. Administration of asPNA to cultures derived from the WT or *msi1*<sup>-/-</sup> embryonic forebrain led to a specific and marked reduction in *msi2* expression at both the mRNA and protein levels, as demonstrated by semiquantitative RT-PCR analysis and immunocytochemical detection with the anti-Msi2 antibody (Fig. 4B and C). With the concentrations of the asPNA used in this series of experiments, rapid cellular changes such as nuclear condensation were not observed in most of the individual dissociated cells, confirming that there was no or very little cytotoxicity associated with the asPNA. Homology search with this asPNA sequence against the GenBank/EST or the Celera Discovery System and Celera Genomics' associated databases indicated that any other mRNAs did not contain a completely matched sequence to the designed sequence of the *msi2* asPNA. We performed the control RT-PCR analysis of the organic cation transporter *NKT* (GenBank accession no. U52842), which was expressed in the neurospheres and shared a sequence similarity to asPNA with a two-base mismatch. As shown in Fig. 4C, the asPNA treatment had no effect on the level of *NKT* mRNA expression in the *msi1*<sup>-/-</sup> or littermate cultures, confirming the target specificity of asPNA. In the neurosphere formation assay, the presence of the asPNA caused a drastic reduction in the number of neurospheres formed in the *msi1*<sup>-/-</sup> cultures in a dose-dependent manner (Fig. 4F). In contrast, the greatest concentration of asPNA used in our study did not decrease the number of neurospheres formed in the WT cultures, indicating that the suppression of Msi2 alone did not affect the neurosphere-forming ability and viability of CNS stem cells. Administration of the nonspecific scrambled PNA did not affect the number of neurospheres formed in both the WT and *msi1*<sup>-/-</sup> cultures (Fig. 4E), excluding the possibility that the *msi1*<sup>-/-</sup> CNS stem cells are more susceptible to PNA-mediated cytotoxicity than WT cells. We, therefore, concluded that the reduction of neurosphere formation mediated by asPNA is a specific effect and that a cooperative action of both Msi1 and Msi2 is essential for the proliferation and/or maintenance of embryonic CNS stem cells.

We sought to establish the characteristics of *msi1*<sup>-/-</sup> CNS stem cells that exposed to the asPNA. Although there are no unambiguous markers for CNS stem cells, we analyzed the number of cells that express nestin, a marker for neural progenitor cells including CNS stem cells (1). Dissociated cells from the primary neurospheres were incubated with the asPNA during the first 24 h before the majority of the SFCs started cell division. Immunocytochemical analysis indicated that nestin<sup>+</sup> cells were ~55% of total cells in WT and *msi1*<sup>-/-</sup> cells, and the number of nestin<sup>+</sup> cells were unchanged with or without asPNA treatment (Fig. 4G), suggesting that the asPNA treatment did not influence the nestin expression and that there were the equivalent number of progenitor cells at the beginning of their sphere formation. Next, we determined the effect of asPNA on the proliferation of neural progenitor cells including CNS stem cells. BrdU was administered into the sphere cultures that have pretreated with asPNA for 2 div. Twenty-four hours later (3 div), the individual forming neurospheres, which were in a phase of the exponential proliferation of SFCs, were harvested, and the BrdU-labeled cells were counted. Without the asPNA treatment, the labeling index for BrdU was unchanged in *msi1*<sup>-/-</sup> neurospheres relative to the WT (~75% of total cells); however, the marked reduction of BrdU-labeled cells was evident in the asPNA-treated *msi1*<sup>-/-</sup> neurospheres (~40% of total cells) (Fig. 4H). Neurosphere differentiation assay revealed that the asPNA treatment did not lead a significant change in the differentiation ability in the *msi1*<sup>-/-</sup> SFCs (Fig. 4I). Taken together, the reduction of neurosphere formation in *msi1*/*msi2* functional double knockout cells was primarily attributable to the inhibition of the SFC

proliferation, rather than due to an aberrant cell-fate choice. Because the short exposure of the asPNA (1 div) seemed to be unaffected for the number of nestin<sup>+</sup> cells, *msi1* and *msi2* may not be involved directly in a survival of the neural progenitor cells including CNS stem cells.

Among the numerous RNA-binding proteins, Msi2 and Msi1 form a unique subgroup in the hnRNP A/B class that contains two copies of RRM. Within this class, the Msi family has a sequence similarity to AUF1 (hnRNP D) and hnRNP A1, albeit to a lesser degree (4). To assess whether the expression levels of these *msi*-related genes altered in the functional double knockout cells, we performed a semiquantitative RT-PCR analysis. A significant up-regulation of the mRNA for AUF1 was detected in the asPNA-treated *msi1*<sup>-/-</sup> cultures without affecting the expression levels of hnRNP A1 and hnRNP C1/C2 (Fig. 4C).

## Discussion

Here, we showed that disruption of the *msi1* gene causes hydrocephalus. The abnormal proliferation and/or differentiation of ependymal cells lining the aqueduct may be the primary cause of the obstruction of the CSF passage, which results in the perinatal onset of hydrocephalus in *msi1*<sup>-/-</sup> mice. However, we found no significant difference in the total number of ependymal cells lining the whole area of the aqueduct in perinatal *msi1*<sup>-/-</sup> pups, as determined by counting the cells in serial sections (not shown). In addition, the BrdU pulse-labeling experiments at P3 and P10 brains showed that there was no difference in the total number of proliferating cells along the aqueductal ependyma between *msi1*<sup>-/-</sup> pups and their WT littermates (not shown), in contradiction to the stratification and polyposis formation in the postnatal SCO. These morphologically abnormal ependymal cells were rarely labeled with BrdU at least by 4 h of BrdU labeling at P3 (not shown). They may proliferate slower than other normal ependymal populations during the aqueductal development. Collectively, Msi1 is likely to be essential for the differentiation or proper functions of the highly restricted cell populations in the ependyma lining the aqueduct. Conceivably, these cells may have a higher susceptibility to the deficiency of the *msi1* gene than do the ependymal cells located in other regions of the ventricular system. Nevertheless, considering the coexpression of Msi1 and Msi2 in most ependymal populations (Fig. 2N), it remains unclear why the defects in *msi1*<sup>-/-</sup> mice are restricted to a certain population of ependymal cells. Msi1 may have a specific function that is required for the development of aqueductal ependymal cells.

The CNS stem cells continue to proliferate in an undifferentiated state (self-renewal proliferation), and also can give rise to the lineage-restricted progenitors, i.e., the neuronal or glial progenitors. The antisense ablation experiments of the *msi2* gene indicated the vital functions cooperated with the *msi* genes during the formation of the neurosphere. Functionally double knockout neurosphere cells showed the reduction of proliferation. This finding suggests that two *msi* genes are involved in the self-renewal proliferation of CNS stem cells. Alternatively, such phenomena could have been caused by enhanced differentiation of CNS stem cells to lineage-restricted progenitors. However, the differentiation potential of double knockout cells was not significantly changed from that of *msi1*<sup>-/-</sup> cells, suggesting that the *msi2* gene is not essential for the lineage restriction of CNS stem cells.

Recent *in vitro* and *in vivo* studies have provided intriguing evidence regarding a source of CNS stem cells in the postnatal CNS. Johansson *et al.* (15) showed that CNS stem cells could originate from ependymal cells in the adult brain and spinal cord, whereas Doetsch *et al.* (16) provided compelling evidence that SVZ astrocytes are precursors for neurogenesis and could possibly serve as stem cells. Although it has been controversial which type of cells serves as the source of postnatal CNS stem

cells *in vivo*, the disorganized proliferation or premature differentiation of *msi1*<sup>-/-</sup> ependymal cells may reflect an impairment of the proliferation or maintenance of CNS stem cells residing in the aqueductal ependyma. However, this idea regarding the ependymal proliferation caused by the loss of *msi1* seemed to be inconsistent with the proposed function of *msi* genes that positively regulate the proliferation of CNS stem cells. It is possible that there is a unique function of the *msi1* gene that keeps the aqueductal ependymal cells quiescent that the *msi2* gene cannot compensate for. Alternatively, the *msi2* gene may be functionally "overexpressed" in the absence of *msi1* in the restricted aqueductal ependymal cell population. Because the Msi family proteins bind to a similar RNA sequence, excessive binding of Msi2 protein to the target mRNAs normally occupied by the Msi1 protein could account for the ependymal proliferation through the misregulation of target mRNAs. Such ependymal proliferation might be implicated in the genesis of ependymal tumors, ependymomas. In fact, of the various brain tumors, the expression levels of the Msi family proteins are markedly elevated in ependymoma cells (unpublished results). Msi1 expression also correlates well with the proliferative activity and malignancy of human gliomas (6), suggesting the involvement of deregulated Msi1 expression in tumorigenesis. Appropriately and tightly regulated expression levels of the *msi* family genes may be crucial not only for the proper development of neural precursors but also to prevent the clonal expansion of proliferative tumors.

Msi1 and Msi2 show a marked sequence similarity, especially within their RRM domains (~90%), which are responsible for specific binding with target RNA molecules. Our previous *in vitro* RNA-binding assay (4) revealed that Msi1 and Msi2 have a very similar RNA-binding specificity characterized by uridine-rich sequences. The cooperative and redundant functions of *msi2* and *msi1* in CNS stem cells further support the idea that Msi1 and Msi2 interact with common target RNAs *in vivo*. In addition, our present RT-PCR analysis indicated the up-regulation of AUF1 mRNA in the asPNA-treated *msi1*<sup>-/-</sup> cultures. AUF1 is

a key regulatory factor of gene expression that is known to bind uridine-rich elements of 3'-UTRs and regulate the stability of many protooncogene and cytokine mRNAs (17). It is unclear whether the Msi proteins and AUF1 share the common downstream target mRNAs. Therefore, we could not exclude a possibility that the loss of two *msi* gene activities could have been compensated by the enhanced expression of AUF1 in the neurospheres derived from the *msi2* asPNA-treated *msi1*<sup>-/-</sup> cells (Fig. 4F). The molecular mechanism underlying the functions of the Msi family should be elucidated by identifying their target mRNAs. One of the candidates is the mRNA of mammalian *numb* (*m-numb*), which encodes a membrane-associated antagonist of Notch signaling (18, 19). The Notch signal is required for the self-renewing activity of mammalian CNS stem cells (7, 20, 21). Our recent study (22) indicated that Msi1 binds to the uridine-rich sequence in the 3'-UTR region of *m-numb* mRNA *in vitro* and can repress the expression of *m-numb* at the level of translation in NIH 3T3 cells. These results, combined with the observation that overexpressed Msi1 activates Notch signaling (22), indicate that the Msi family may contribute to the self-renewing activity of CNS stem cells by modulating the *m-numb*-Notch signaling cascade. However, our immunohistochemical study with an anti-*m-Numb* antibody failed to demonstrate a significant increase in the *m-Numb* protein in the neurospheres or brain of *msi1*<sup>-/-</sup> mice (not shown). Considering that the up-regulated *msi2* acts as a redundant gene in the *msi1*<sup>-/-</sup> CNS, loss-of-function experiments with both the *msi2* and *msi1* genes may unequivocally reveal the function of the Msi proteins in mammalian CNS development. We have generated a homozygous mutant harboring a disrupted *msi2* allele, and the phenotypic analyses of double-knockout mice with an *msi2/msi1* genotype are currently in progress in our laboratory.

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(54) **ENGRAFTABLE HUMAN NEURAL STEM CELLS**

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(73) Assignees: **The Children's of Medical Center Corporation**, Boston, MA (US); **The University of British Columbia** (CA); **The University of Pennsylvania**, Philadelphia, PA (US)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/398,298**

(22) Filed: **Sep. 20, 1999**

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(63) Continuation of application No. 09/133,873, filed on Aug. 14, 1998, now Pat. No. 5,958,767.

(51) Int. Cl.<sup>7</sup> ..... **C12N 5/10**

(52) U.S. Cl. .... **435/368; 435/455**

(58) Field of Search ..... **435/368, 455, 435/366, 325**

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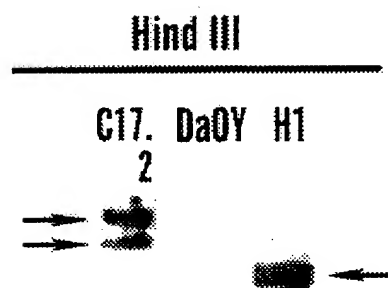
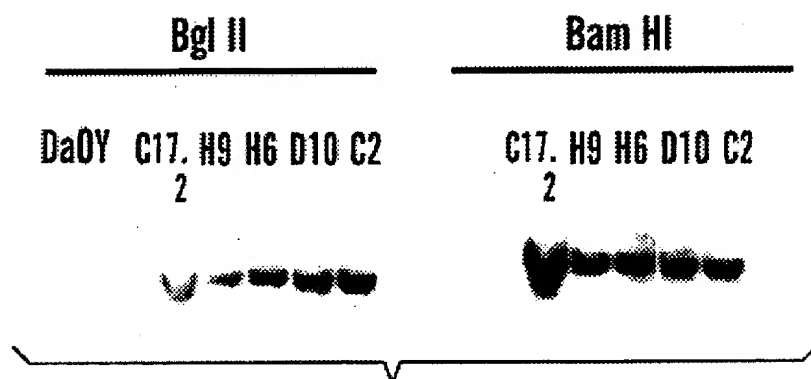
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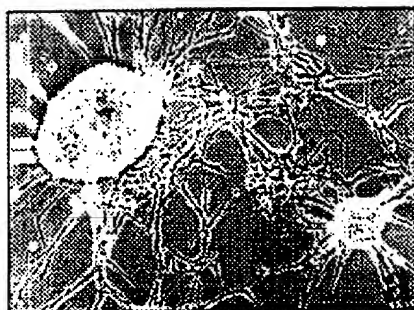
(74) Attorney, Agent, or Firm—Nixon Peabody LLP

(57) **ABSTRACT**

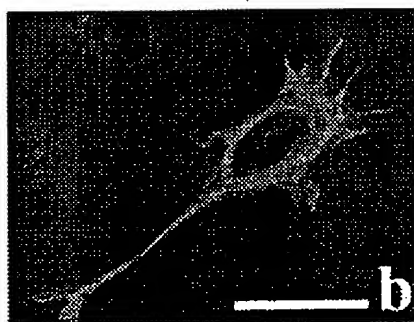
Stable clones of neural stem cells (NSCs) have been isolated from the human fetal telencephalon. In vitro, these self-renewing clones (affirmed by retroviral insertion site) can spontaneously give rise to all 3 fundamental neural cell types (neurons, oligodendrocytes, astrocytes). Following transplantation into germinal zones of the developing newborn mouse brain, they, like their rodent counterparts, can participate in aspects of normal development, including migration along well-established migratory pathways to disseminated CNS regions, differentiation into multiple developmentally- and regionally-appropriate cell types in response to microenvironmental cues, and non-disruptive, non-tumorigenic interspersions with host progenitors and their progeny. Readily genetically engineered prior to transplantation, human NSCs are capable of expressing foreign transgenes in vivo in these disseminated locations. Further supporting their potential for gene therapeutic applications, the secretory products from these NSCs can cross-correct a prototypical genetic metabolic defect in abnormal neurons and glia in vitro as effectively as do murine NSCs. Finally, human cells appear capable of replacing specific deficient neuronal populations in a mouse model of neurodegeneration and impaired development, much as murine NSCs could. Human NSCs may be propagated by a variety of means—both epigenetic (e.g., chronic mitogen exposure) and genetic (transduction of the propagating gene *vmyc*)—that are comparably safe (*vmyc* is constitutively downregulated by normal developmental mechanisms and environmental cues) and effective in yielding engraftable, migratory clones, suggesting that investigators may choose the propagation technique that best serves the demands of a particular research or clinical problem. All clones can be cryopreserved and transplanted into multiple hosts in multiple settings.

**3 Claims, 6 Drawing Sheets**

**FIG. 1A****FIG. 1B**



**FIG. 2A**



**FIG. 2B**



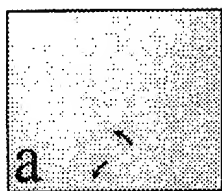
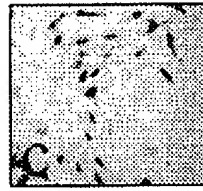
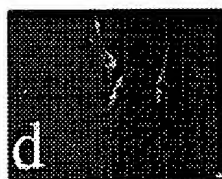
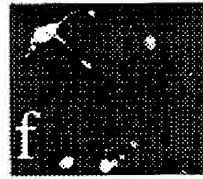
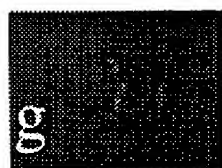
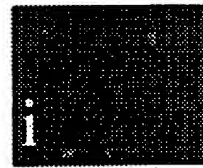
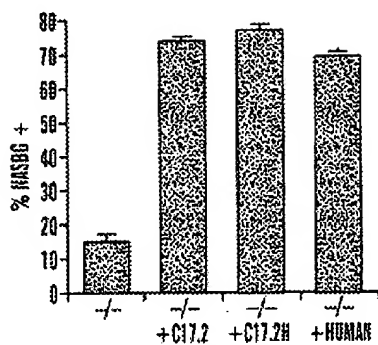
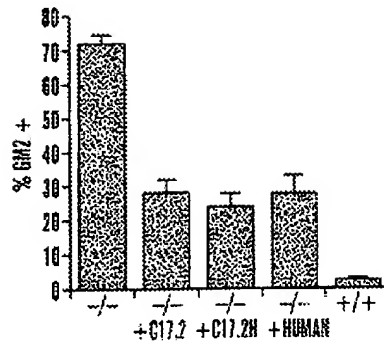
**FIG. 2C**

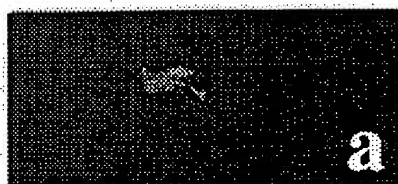
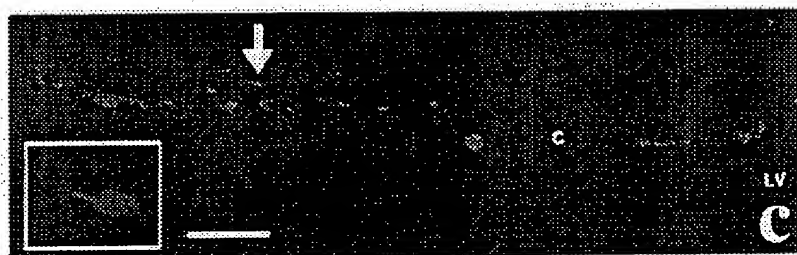
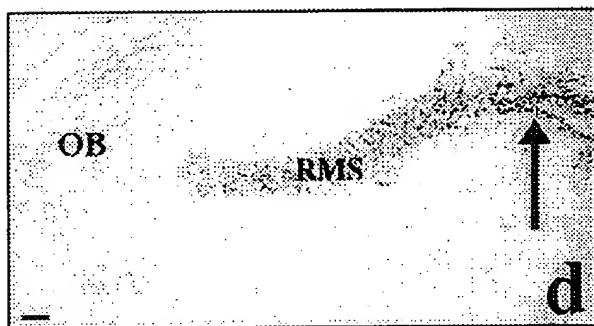


**FIG. 2D**

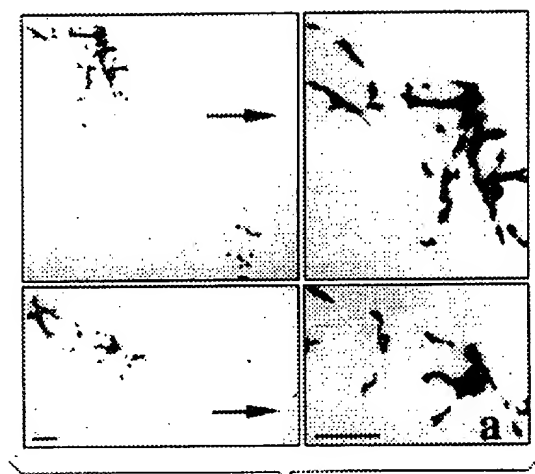
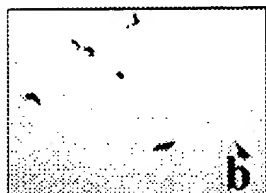
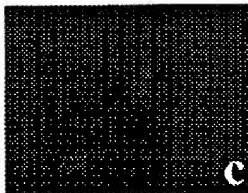
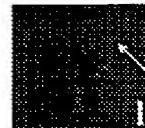


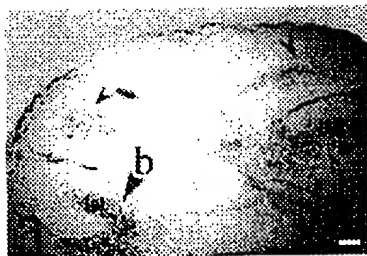
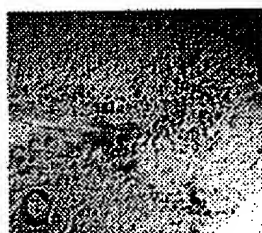
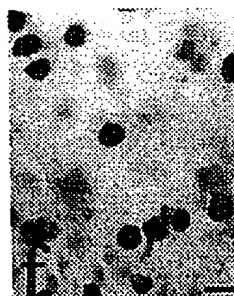
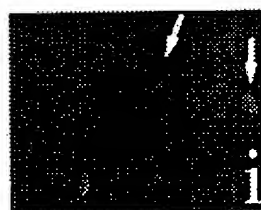
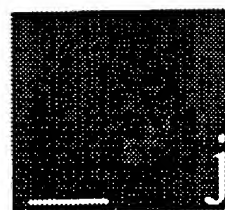
**FIG. 2E**

**FIG. 3A****FIG. 3B****FIG. 3C****FIG. 3D****FIG. 3E****FIG. 3F****FIG. 3G****FIG. 3H****FIG. 3I****FIG. 3J****FIG. 3K****FIG. 3L****FIG. 3M****FIG. 3N**

**FIG. 4A****FIG. 4B****FIG. 4C****FIG. 4D****FIG. 4E**



**FIG. 5A****FIG. 5D****FIG. 5E****FIG. 5F****FIG. 5B****FIG. 5C****FIG. 5G****FIG. 5H****FIG. 5I****FIG. 5K****FIG. 5L****FIG. 5J****FIG. 5M****FIG. 5N****FIG. 5O****FIG. 5P****FIG. 5Q**

**FIG. 6A****FIG. 6B****FIG. 6C****FIG. 6D****FIG. 6E****FIG. 6F****FIG. 6G****FIG. 6H****FIG. 6I****FIG. 6J**

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## ENGRAFTABLE HUMAN NEURAL STEM CELLS

This is a continuation of Application Ser. No. 09/133,873 filed on Aug. 14, 1998, now U.S. Pat. No. 5,958,767.

Neural stem cells (NSCs) are postulated to be relatively primordial, uncommitted cells that exist in the developing and even adult nervous system and are responsible for giving rise to the array of more specialized cells of the mature CNS<sup>1-12</sup>. They are operationally defined by their ability (a) to differentiate into cells of all neural lineages (neurons—ideally of multiple subtypes, oligodendroglia, astroglia) in multiple regional and developmental contexts (i.e., be multipotent); (b) to self-renew (i.e., also give rise to new NSCs with similar potential); (c) to populate developing and/or degenerating CNS regions. An unambiguous demonstration of monoclonal derivation is obligatory to the definition—i.e., a single cell must possess these attributes. With the earliest recognition that rodent neural cells with stem cell properties, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes<sup>13-16</sup>, gene therapists and restorative neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies which they spawned (reviewed elsewhere<sup>2,9-11,17-21</sup>) provided hope that the use of NSCs—by virtue of their inherent biology—might circumvent some of the limitations of presently available graft material and gene transfer vehicles and make feasible a variety of novel therapeutic strategies<sup>20-22</sup>. Neural cells with stem cell properties have been isolated from the embryonic, neonatal and adult rodent CNS and propagated in vitro by a variety of equally effective and safe means—both epigenetic (e.g., with mitogens such as epidermal growth factor [EGF] or basic fibroblast growth factor [bFGF]<sup>1,5,16,23-27</sup> or with membrane substrates<sup>7</sup>) and genetic (e.g., with propagating genes such as vmyc or SV40 large T-antigen<sup>1,9-15,17-19,28-32</sup>). Maintaining such NSCs in a proliferative state in culture does not appear to subvert their ability to respond to normal developmental cues in vivo following transplantation—to withdraw from the cell cycle, interact with host cells, differentiate appropriately<sup>9-16,29-33</sup>. These extremely plastic cells migrate and differentiate in a temporally and regionally appropriate manner particularly following implantation into germinal zones throughout the brain. They participate in normal development along the murine neuraxis, intermingling non-disruptively with endogenous progenitors, responding similarly to local microenvironmental cues for their phenotypic determination and appropriately differentiating into diverse neuronal and glial cell types. In addition, they can express foreign genes (both reporter genes and therapeutic genes) in vivo<sup>9-21,29-32</sup>, and are capable of specific neural cell replacement in the setting of absence or degeneration of neurons and/or glia<sup>9,11,31,32</sup>.

### SUMMARY OF THE INVENTION

This paper affirms the potential of clones of human NSCs to perform these critical functions in vitro and in vivo. We show them to be multipotent, self-renewing, engraftable, plastic, and migratory; to secrete gene products that can cross-correct a prototypical neurodegenerative genetic enzymatic defect (a necessary precursor for gene therapy of such diseases); to pursue in vivo (following transplantation into various germinal zones) developmental programs appropriate to a given region and stage (even if different from that

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from which the cells were initially obtained); to be capable of ex vivo genetic manipulation—e.g., retroviral-mediated transduction of a foreign gene—and to be capable, following transplantation, of expressing that transgene in vivo in widely disseminated CNS regions (further establishing gene delivery/therapy potential); and to be capable of differentiating towards replacement of specific deficient neuronal populations in a prototypic mouse mutant model of neurodegeneration and impaired development (suggesting a potential for therapeutic CNS cell replacement). Comparison is also made between the capabilities of human NSCs propagated by either epigenetic (with bFGF) or genetic (via a constitutively downregulated vmyc) means; the findings suggest that these two common means of propagation are equally effective and safe (inferring that investigators may freely choose the technique that best fits their research or clinical demands.)

### DETAILED DESCRIPTION OF THE INVENTION

We present evidence that neural cells with rigorously defined stem cell features, may, indeed, be isolated from the human brain and may emulate the behavior of NSCs in lower mammals. Not only do these observations vouchsafe conservation of certain neurodevelopmental principles to the human CNS, but they suggest that this class of neural cells may ultimately be applied as well to research and clinical problems in the human. Indeed, not only might the actual human NSC clones described in this report serve that function, but our data suggest that other investigators may readily obtain and propagate such cells from other sources of human material through a variety of equally safe and effective methods (both epigenetic and genetic) with the expectation that such cells will fulfill the demands of multiple research and/or therapeutic problems.

The growing interest in the potential of NSCs has been analogous to that in hematopoietic stem cells. This interest derives from the realization that NSCs are not simply a substitute for fetal tissue in transplantation paradigms or simply another vehicle for gene delivery. Their basic biology, at least as revealed through the examination of cells, appears to endow them with a potential that other vehicles for gene therapy and repair may not possess. For example, that they may integrate into neural structures after transplantation may allow for the regulated release of various gene products as well for literal cell replacement. (While presently available gene transfer vectors usually depend on relaying new genetic information through established neural circuits, which may, in fact, have degenerated and require replacement, NSCs may participate in the reconstitution of these pathways.) The replacement of enzymes and of cells may not only be targeted to specific, anatomically circumscribed regions of CNS, but also, if desired, to larger areas of the CNS in a widespread manner by simple implantation into germinal zones. This ability is important because many neurologic diseases are not localized to specific sites as is Parkinson's disease. Rather their neuropathology is often extensive, multifocal, or even global (e.g., the lesions present in various traumatic, immunologic, infectious, ischemic, genetic, metabolic, or neurodegenerative processes). These are therapeutic challenges conventionally regarded as beyond the purview of neural transplantation. NSCs, therefore, have helped to broaden the paradigmatic scope of transplantation and gene therapy in the CNS. NSCs pass readily and unimpeded through the blood-brain barrier and deliver their foreign gene products immediately, directly, and, if necessary, in a disseminated fashion to the

CNS. In addition, NSCs may be responsive to neurodegeneration, shifting their differentiation to compensate for deficient cell types. The biology underlying these properties may not only be of practical value but might illuminate fundamental developmental mechanisms.

To summarize our results, clones of human NSCs—unambiguously affirmed by the presence of a common retroviral insertion site and propagated by either epigenetic or genetic means—can participate in normal CNS development *in vivo* and respond to normal microenvironmental cues, including migration from various germinal zones along well-established migratory routes to widely disseminated regions. A single NSC is capable of giving rise to progeny in all 3 fundamental neural lineages—neurons (of various types), oligodendroglia, and astroglia (hence, multipotency)—as well as giving rise to new NSCs with similar potential (i.e., self-renewal). *In vivo*, following transplantation into mouse hosts, a given human NSC clone is sufficiently plastic to differentiate into neural cells of region- and developmental stage-appropriate lineages along the length of the neural axis: into neurons where neurogenesis normally persists, and into glia where gliogenesis predominates, emulating patterns well-established for endogenous murine progenitors, with which they intermingle seamlessly. Thus, for example, they will give rise to neurons following migration into the OB at one end of the neuraxis and into granule neurons in the cerebellum at the other, yet also yield astroglia and oligodendroglia, the appropriate cell types born in the postnatal neocortex, subcortical white matter, and striatum. Of additional significance, as might be expected of a true stem cell, we could demonstrate that many of the neuronal types into which these NSCs could differentiate, are born not at the developmental stage from which the cells were initially obtained (e.g. midgestation), but rather at the stage and region of NSC implantation, thus affirming appropriate temporal (in addition to regional) developmental responsiveness.

Interestingly, the most robust differentiation was ultimately not achieved in the culture dish where cells could maintain a more undifferentiated appearance for prolonged periods, but in the transplanted brain, where they rapidly pursued differentiated phenotypes. This conclusion is also supported by the observation that, for the *in vitro* experiments, triggering astroglialogenesis (the last cell type typically born in the developing brain) required the presence of co-cultured primary CNS cells (presumably recreating the “milieu”) whereas simple implantation of the NSCs into the *in vivo* environment was sufficient in the transplant experiments.

Such abundant, genetically-homogeneous, manipulatable cells clearly represent a valuable model for studying human NSC biology *in vivo* and *in vitro*. In this paper, we have demonstrated that human NSC clones possess the capabilities that might lead one to expect them to be effective in true clinician situations.

We demonstrate the ability of these cells, in their widely disseminated locations (from even a single, simple implantation procedure) to express a retrovirally-transduced foreign gene (*lacZ*), providing promise for future therapeutic gene transfer strategies. That gene products delivered by these human NSCs might be expected to cross-correct dysfunctional neural cells of all types was suggested by our experiments demonstrating the successful delivery of an index therapeutic protein (hexosaminidase) to a prototypical model of neural cells deficient (via targeted mutagenesis) in that specific gene product (Tay-Sachs mouse cells). Tay-Sachs brain cells of neuronal, glial, and even immature

neuroepithelial progenitor phenotypes could be effectively rescued by the secretory products of these human NSCs and complement them effectively. Once internalized in the target neural cells, this gene product forestalled pathologic GM<sub>2</sub> accumulation in the majority of mutated cells. This successful molecular cross-correction taken together with the cellular transplantation and *in vivo* migration data help establish the feasibility of human NSC-mediated strategies for the treatment of extensive inherited metabolic and other neuro-genetic human diseases for which Tay-Sachs is an exemplar.

In summary, NSCs may be propagated by a variety of means (both epigenetic and genetic) that are comparably effective and safe in yielding engraftable, responsive neural cells (and may, in fact, access common final molecular pathways that interact reversibly with cell cycle regulatory proteins). Therefore, insights gained from studies of NSCs perpetuated by one technique may be legitimately joined with insights derived from studies employing others to help provide a more complete picture of NSC biology. Furthermore, in helping to resolve debate in the NSC literature as to which techniques are most effective for isolating and manipulating NSCs, and doing so with cells of human origin, the door is open for investigators and/or clinicians to pick the propagation technique that best serves the demands of their particular research or clinical problem. These may have significant practical implications. It is interesting that propagating NSCs by genetic means (e.g. a *vmc* construct that is constitutively downregulated by normal developmental mechanisms and environmental cues) appears to be among the safest, easiest, most efficacious, reliable, and cost-effective methods to date for many needs.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and B: The monoclonal nature of each putative human neural stem cell (NSC) clone is confirmed by demonstrating a single retroviral insertion site within the genomes of each. [A] Genomic DNA from the putative human NSC clone H1 (which was propagated in bFGF and subsequently transduced with a retrovirus encoding *lacZ* and *neo*) was digested with Hind III (which cuts only once within the provirus) and incubated with a radiolabeled nucleotide probe complementary to *neo*. Monoclonal derivation is confirmed by the presence of a single integrated retrovirus with an integration site common to all cells in the colony indicating that they were derived from a single infected “parent” cell (arrow). As a positive control, the murine NSC clone C17.2 which contains 2 integrated retroviruses encoding *neo* (one from an integrated *vmc*-encoding retrovirus and one from a separate *lacZ*-encoding retrovirus<sup>13,28</sup>) appropriately shows 2 bands (arrows). Specificity of the probe is demonstrated by the negative control, the human medulloblastoma cell line DaOY, which, having not been infected with a retrovirus, shows no *neo* sequences in its genome and hence no hybridization product. [B] Genomic DNA from putative clones H9, H6, D10, and C2 (human NSC colonies propagated in bFGF and/or EGF and then subsequently infected with a retrovirus encoding the propagating gene *vmc*) were digested with Bgl II or Bam HI (each of which cuts only once within the provirus) and then subjected to Southern analysis utilizing a probe complementary to the proviral *vmc*. Single retroviral integration sites are appreciated in all colonies confirming the monoclonal nature of each putative clone. The murine NSC clone C17.2, which contains a single copy of *vmc*<sup>13,28</sup> and serves as a positive control, also has one band. As in [A], the negative control non-virally infected human DaOY cells, have no bands.

FIGS. 2A–2E: Characterization of human neural stem cells (NSCs) *in vitro*. [A] NSCs tend to grow as clusters in serum-free bFGF-supplemented medium. They differentiate spontaneously into neurofilament-immunoreactive neurons [B] or CNPase-immunoreactive oligodendrocytes [C] when transferred to serum-containing medium, or into GFAP-expressing astrocytes when co-cultured with primary murine CNS cultures (and identified with a human-specific anti-GFAP antibody as, for example in [D], illustrating a typical type-1 protoplasmic astrocyte. Hence, a single one has the potential for generating cells of all neural lineages (“multipotency”). New immature, undifferentiated, vimentin-immunoreactive NSCs [E] are present in clones under all conditions, suggesting the ability of a clone to “self-renew” (i.e., produce new multipotent NSCs).

FIGS. 3A–3N: Human neural stem cells (NSCs) are capable of complementing a prototypical gene product deficiency (e.g.,  $\beta$ -hexosaminidase-A) in neural cells of multiple lineages in which the gene is mutated (e.g., brain cells from Tay-Sachs mice). As a proof of principle that human NSCs (like murine NSCs) are capable of cross-correcting a neurogenetic defect, neural cells from the brains of mice with the prototypical neurogenetic disorder Tay-Sachs disease, generated via targeted mutagenesis of the  $\alpha$ -subunit of  $\beta$ -hexosaminidase resulting in absence of hexosaminidase-A<sup>39</sup>, were exposed to secreted gene products from human NSCs to assess their ability to effect complementation of the defect. [A–C] Hexosaminidase activity as determined by NASBG histochemistry (Nomarski optics). Functional hexosaminidase produces a red-pink precipitate with an intensity proportional to the level of activity. [A] Tay-Sachs neural cells (arrows) not exposed to NSCs have no, or minimal, detectable hexosaminidase. (A small number of faintly pink NASBG+ cells are occasionally observed reflecting low residual hexosaminidase-B activity). In comparison, Tay-Sachs neural cells exposed to secretory products from murine NSCs (e.g., clone C17.2H) [B] or from human NSCs [C] now stain intensely red (wildtype intensity) suggesting that they have been cross-corrected, i.e., have internalized significant amounts of functionally active hexosaminidase from the NSC-conditioned medium. [D–L] To help determine which neural cell types from the Tay-Sachs brain were cross-corrected, primary dissociated Tay-Sachs neural cells which had been co-cultured in a transwell system with human NSCs (as in [C]) were reacted both with a fluorescein-labeled antibody to the human  $\alpha$ -subunit of hexosaminidase [D–F] and with antibodies to neural cell type-specific antigens (visualized by a TR-tagged secondary antibody) [G–I, respectively]. Photomicroscopy through a dual filter confirmed co-localization of the  $\alpha$ -subunit with the cell-type markers [J–L, respectively]. A subset of these now  $\alpha$ -subunit-positive corrected cells [D] were neurons, as indicated by their expression of the neuronal marker NeuN [G,J]; a subset of the  $\alpha$ -subunit+ cells [E] were glial as illustrated by their co-expression of the glial marker GFAP [H,K]; and a subset of the  $\alpha$ -subunit+ cells [F] were immature, undifferentiated CNS precursors, as indicated by the presence of the intermediate filament nestin [I,L]. (Untreated cells from a Tay-Sachs brain do not stain for the  $\alpha$ -subunit). [M] Percentage of successfully rescued (i.e., NASBG+) primary Tay-Sachs neural cells as seen in [A–C]. The number of “untreated” Tay-Sachs  $\alpha$ -subunit-null cells (–/–) (i.e., unexposed to NSCs) that were NASBG+ (1st histogram) was quite low. (That the percentage is not 0 reflects some low residual hexosaminidase-B activity in mutant cells that is sometimes sufficient enough in some cells to produce a pale pink scoreable cell). In contrast,

among Tay-Sachs neural cells “treated” with secretory products from murine NSCs (C17.2) (2nd histogram), murine NSCs engineered to over-express hexosaminidase (C17.2H) (3rd histogram), or human NSCs (4th histogram), the percentage of cross-corrected, hexosaminidase-containing cells was significantly increased ( $p < 0.01$ ). The NSCs did not significantly differ from each other in their ability to effect this rescue. (NASBG staining of neural cells from a wildtype mouse served as a positive control and were nearly 100% NASBG+, histogram not presented). [N] Complementation of gene product deficiency results in rescue of a pathologic phenotype in mutated neural cells, as illustrated by percentage of Tay-Sachs CNS cells with diminished GM<sub>2</sub> accumulation. Among Tay-Sachs cells not exposed to NSCs (1st histogram), the percentage of GM<sub>2</sub>+ cells was large reflecting their pathologically high level of storage and consistent with a lack of enzyme as per [M]. In contrast, the percentage of cross-corrected Tay-Sachs cells without detectable GM<sub>2</sub> storage following exposure to murine (2nd and 3rd histograms, as in [M]) or human NSCs (4th histogram) was significantly lower than in the mutant ( $p < 0.01$ ), approaching that in wildtype (+/+) mouse brain (5th histogram). Again, the NSCs did not significantly differ from each other in their ability to effect this rescue.

FIGS. 4A–4E: Developmentally-appropriate migration of human neural stem cells (NSCs) following engraftment into the subventricular germinal zone (SVZ) of newborn mice. [A,B] Donor-derived human NSCs integrate and intermingle non-disruptively with endogenous progenitors within the host SVZ by 24 hours after transplantation. A representative donor-derived cell with a typical short process (highlighted in [A]), has interspersed with densely packed endogenous SVZ cells, visualized by DAPI (blue) in the overlapping image in [B]. [C] Two weeks following transplantation, many donor-derived cells (red) have migrated extensively within the subcortical white matter (arrow) and corpus callosum (c) from their site of implantation in the lateral ventricles (LV), as visualized in this coronal section. A representative migrating cell within the subcortical white matter (arrow), visualized at higher magnification in the boxed insert, is noted to have a leading process characteristic of migrating precursor cells. [D,E] As seen in this representative cresyl violet-counterstained parasagittal section, other donor-derived cells migrated from their integration site in the anterior SVZ to enter the rostral migratory stream (“RMS”) leading to the olfactory bulb (“OB”). Representative BrdU-immunoperoxidase-positive (brown) donor-derived cells (arrow) within the RMS, are seen at low power in [D] and visualized at higher magnification in [E], intermingled with migrating host cells. Further characterization and visualization of these donor human NSC-derived cells in their final location in the OB are presented in FIG. 5. Scale Bars: 100  $\mu$ m.

FIGS. 5A–5Q: Differentiation and disseminated foreign gene ( $\beta$ -galactosidase) expression of human neural stem cell (NSC) clones *in vivo* following engraftment into the SVZ of developing, neonatal mice. [A–C] Stably engrafted,  $\beta$ -galactosidase ( $\beta$ gal)-expressing, donor-derived cells from representative human NSC clone H1, detected with Xgal histochemistry [A,B] and with anti- $\beta$ gal ICC [C]. The donor-derived cells pictured in the series of photomicrographs in [A] are within the periventricular and subcortical white matter regions (as per FIG. 4). (The top and bottom panels—low power on the left, corresponding high power on the right—are from representative semi-adjacent regions within a single recipient, suggesting a significant distribution of cells; arrows indicate the lateral ventricles).

Furthermore, as illustrated in [B,C] by representative high power photomicrographs through the olfactory bulb (OB) (located as in FIG. 4D), donor-derived cells from this clone have not only migrated extensively to this developmentally-appropriate site, but continue to express  $\beta$ gal in this distant location (i.e., in a disseminated fashion *in vivo*). The normal fate of a subpopulation of SVZ-derived progenitors that have migrated to the OB at this developmental stage is to become neuronal.

In [D-G], donor-derived neurons in the mature OB, derived from BrdU-labeled NSCs (representative clone H6) implanted into the SVZ at birth, are identified by both their immunoreactivity to a human-specific NF antibody [D] as well as their expression of the mature neuronal marker, NeuN [E-G]; under confocal microscopy, a BrdU+ (hence, donor-derived) cell (arrow in [E], fluorescein) is NeuN+ (arrow in [F], Texas Red) appreciated best with a dual filter (arrow in [G]). Adjacent to this representative donor-derived BrdU+/NeuN+ neuron (arrow), are 2 host OB neurons (BrdU-/NeuN+ in [G]) which share a similar size, morphology, and location with the donor-derived cell [arrow in F]. [H,I] High power view of a representative donor-derived (clone H6) oligodendrocyte (arrow), appropriately in the adult subcortical white matter (as per FIG. 4C) following neonatal intraventricular implantation, double-labeled with an antibody to the oligodendrocyte-specific protein CNPase [H] and BrdU [I]. Characteristic cytoplasmic processes extending from the soma are noted (arrowhead in [H]). (The morphology of the CNPase+cell has been somewhat damaged by the HCl pre-treatment required for BrdU double-labeling). [J] Mature donor-derived astrocytes (clone H6) in the adult subcortical white matter (arrow) (as per FIG. 4C) and striatum following neonatal intraventricular implantation, identified with a human-specific anti-GFAP antibody. The inset better illustrates at higher magnification the characteristic mature astrocytic morphology of a representative human-GFAP+cell. [K-Q] Expression of *vmyc* is downregulated within 48 hours following engraftment. [K], [M], and [O] are DAPI-based nuclear stains of the adjacent panels [L], [N], and [P, Q], respectively. Representative human NSC clone H6 was generated (as was the well-characterized murine NSC clone C17.2) with the propagating gene *vmyc*. *vmyc* immunoreactivity in H6-derived cells (red) in the SVZ (arrows) at 24 hours following engraftment ([L] and at higher power in [N]), is persistently absent [P] in integrated H6-derived cells (visualized by BrdU labeling in [Q]) (shown here 3 weeks following transplantation, but representative of any point 24 hours after engraftment). Scale Bars: [A], [K] and applies to [L]: 100 $\mu$ m; [D], [E] and applies to [F,G], [H] and applies to [I], [J], [M] and applies to [N]: 10 $\mu$ m; [O] and applies to [P,Q]: 50 $\mu$ m

FIGS. 6A-6J: Neuronal replacement by human neural stem cells (NSCs) following transplantation into the cerebellum of the granule neuron-deficient meander tail (mea) mouse model of neurodegeneration. [A-G] BrdU-intercalated, donor-derived cells (from representative clone H6) identified in the mature cerebellum by anti-BrdU immunoperoxidase cytochemistry (brown nuclei) following implantation into the neonatal mea external germinal layer (EGL). (The EGL, on the cerebellar surface, disappears as the internal granule layer (IGL) emerges to become the deepest cerebellar cortical layer at the end of organogenesis<sup>13</sup>) [A] Clone H6-derived cells are present in the IGL ("igl"; arrowheads) of all lobes of the mature cerebellum in this parasagittal section. (Granule neurons are diminished throughout the cerebellum with some promi-

nence in the anterior lobe). [B] Higher magnification of the representative posterior cerebellar lobe indicated by arrowhead "b" in [A], demonstrating the large number of donor-derived cells present within the recipient IGL. [C-G] Increasing magnifications of donor-derived cells (brown nuclei) within the IGL of a mea anterior cerebellar lobe. (Different animal from that in [A,B].) [G] Normarski optics bring out the similarity in size and morphology of the few residual host, BrdU-negative cerebellar granule neurons (arrowheads) and a BrdU+, donor-derived neuron (arrow), which is representative of those seen in all engrafted lobes of all animals. [H,I] Confirmation of the neuronal differentiation of a subpopulation of the donor-derived, BrdU+ cells from [A-G] is illustrated by co-labeling with anti-BrdU [green in H] and the mature neuronal marker NeuN [red in I] (indicated with corresponding arrows). (Some adjacent, donor-derived cells are non-neuronal as indicated by their BrdU+ (arrowhead in [H]) but NeuN-phenotype (also illustrating the specificity of the immunostaining). [J] Cells within the IGL are confirmed to be human donor-derived cells by FISH with a human-specific probe (red) identifying human chromosomal centromeres. Scale Bars: [A], [B]: 100 $\mu$ m; [F], [G], [J]: 10 $\mu$ m

#### EXPERIMENTAL PROTOCOL

**Maintenance and Propagation of Human NSCs in Culture.** A suspension of primary dissociated neural cells ( $5 \times 10^5$  cells/ml), prepared from the telencephalon (particularly the periventricular region) of a 15 week gestational human fetus (detailed elsewhere<sup>35</sup>) was plated on uncoated tissue culture dishes (Corning) in the following growth medium: Dulbecco's Modified Eagles Medium (DMEM) plus F12 medium (1:1) supplemented with N2 medium (Gibco) to which was added bFGF (10–20 $\mu$ g/ml) (Calbiochem) + heparin (8 $\mu$ g/ml) and/or EGF (10–20 $\mu$ g/ml). Medium was changed every 5 days. Cell aggregates were dissociated when >10 cell diameters in size as follows: rinsed twice with Hank's balanced salt solution and Hepes buffer, placed in trypsin-EDTA (0.05%) for 15 min. at 37° C., triturated in soybean trypsin inhibitor, pelleted by gentle centrifugation, and replated in growth medium as above at  $5 \times 10^5$  cells/ml.

**Differentiating Culture Conditions.** Dissociated NSCs were plated on poly-L-lysine (PLL)-coated, 8-well chamber slides (Nunc) in DMEM+10% fetal bovine serum (FBS) (Gibco) and processed weekly (up to 4 wks *in vitro*) for ICC. In most cases, differentiation occurred spontaneously. For astrocytic maturation (as assessed by immunoreactivity to a human-specific anti-GFAP antibody), human clones were co-cultured with primary dissociated cultures of newborn CD-1 mouse brain.

**Retrovirus-Mediated Gene Transfer into Human NSCs.** Two different xenotropic, replication-incompetent retroviral vectors were used to infect human NSCs. A retroviral vector encoding lacZ transcribed from the viral long terminal repeat (LTR) plus neo transcribed from an internal SV40 early promoter was similar to the BAG vector<sup>13</sup> except that it bore a PG13 xenotropic envelope. This vector not only provided a stable, histochemically- and immuno-detectable genetic marker for transplantation experiments but also enabled confirmation of monoclonality through the demonstration of a single, common retroviral insertion site on Southern analysis of cells present in a given colony. An amphotropic replication-incompetent retroviral vector encoding *vmyc* (transcribed from the LTR plus neo transcribed from an internal SV40 early promoter) not only permitted the propagation of human NSC clones by genetic means, but also enabled confirmation of the monoclonal

origin of all progeny, as described above. This amphotrophic vector was generated using the ecotropic retroviral vector encoding vmyc (as described for generating the murine NSC clone C17.2<sup>13,28</sup>) to infect the GP+envAM12 amphotrophic packaging lin<sup>54</sup>. Successful infectants were selected and expanded. Supernatants from these new producer cells contained replication-incompetent retroviral particles bearing an amphotrophic envelope at a titer of  $4 \times 10^5$  CFUs which efficiently infected the human neural cells as indicated by G418-resistance. No helper or replication-competent recombinant viral particles were produced. Infection of bFGF- and/or EGF-maintained human neural cells with either vector followed similar procedures: 3ml of supernatant ( $4 \times 10^5$  CFUs) from the respective packaging line were added to suspensions of target cells in 2ml growth medium +polybrene (8  $\mu$ g/ml) and incubated for 4 hrs at 37° C.; the medium was then replaced with fresh growth medium; infection was repeated 24 hrs later, 72 hrs following the second infection, infected cells were selected with G418 (0.3–1.0 mg/ml) for 10 days and individual clones were generated by limiting dilution and propagated as described below. Monoclonality in all cases was confirmed by identifying a single genomic insertion site on Southern analysis for either the lacZ- or the vmyc-encoding virus as described below.

Cloning of Human NSCs. Cells were dissociated as above, diluted to a concentration of 1 cell/15 $\mu$ l in growth medium and plated at 15 $\mu$ l/well of a Terasaki dish. Wells with single cells were noted immediately after plating. Single cell clones were expanded and maintained in bFGF-containing growth medium. Because a retrovirus integrates once randomly into the genome of a host cell with each infection event, its insertion site serves as a unique molecular tag; all progeny of that cell will also bear that and only that tag. Monoclonality in all cases was, therefore, then confirmed by identifying in all progeny a single and identical genomic insertion site on Southern analysis for either the lacZ- or the vmyc-encoding virus. Hybridization to a radiolabeled probe complementary to unique viral sequences yields 1 band at a given molecular weight per retroviral integration site. Probes were generated to either vmyc or to the neo portion of the lacZ-encoding vector as appropriate to the clone. The vmyc probe was generated by nick translation labeling with <sup>32</sup>p dCTP. The neo probe was generated by PCR utilizing <sup>32</sup>p dCTP. Genomic DNA was isolated from the putative human NSC clones by standard procedures and digested with restriction endonucleases that cut only once within the integrated provirus with remaining cuts only in the flanking regions. For analysis with the vmyc probe, DNA was digested with Bgl I or Bam HI; for the neo probe, DNA was cut with Bgl II. The remainder of the Southern analysis followed standard procedures. The non-virally infected DaOY human cell line, which should have no detectable bands, served as a negative control. (To insure the health of clones once generated, particularly when small, 10% FBS and 5% horse serum (HS) were occasionally added to the medium)

Cryopreserving Human NSC Clones. Trypsinized human cells were pelleted and resuspended in a freezing solution composed as follows: 10% DMSO (Sigma), 50% FBS, 40% bFGF-containing growth medium. Divided into 1.5ml Nunc vials, the suspension was slowly brought to -140° C. for long-term storage. Cells were thawed by placing vials in a 37° C. water bath and, following gentle removal from the vial, resuspended and cultured in excess growth medium which was changed initially after 8 hrs. to clear the DMSO.

Cross-Correction of Mutation-Induced  $\beta$ -Hexosaminidase Deficiency. Human NSCs were main-

tained as described above. The murine NSC clones "C17.2" and "C17.2H" (the latter a subclone of C17.2 transduced with a retrovirus encoding the human  $\alpha$ -subunit of  $\beta$ -hexosaminidase<sup>30</sup>), were maintained in similar serum-free conditions. NSCs were co-cultured in a transwell system (as detailed under Results) with primary dissociated neural cultures—from the brains of neonatal mice—either wildtype or  $\alpha$ -subunit null (Tay-Sachs) mice<sup>39</sup>. These primary cultures were prepared under serum-free conditions (trypsin was inactivated with soybean trypsin inhibitor), plated onto PLL-coated 12mm glass coverslips, and maintained in the culture medium described above for NSCs; in one set of control conditions, bFGF+heparin were excluded from the medium; the results were unaffected, however. The presence of hexosaminidase activity was assayed by standard histochemical techniques<sup>30</sup>: Cells were fixed in 4% paraformaldehyde (PFA), washed in 50mM citrate buffer (pH 4.4), and incubated with NASBG (0.25mM) in 50mM citrate buffer (pH4.5) for 3 hrs at 40° C. Subsequently, they were exposed to the same substrate in the presence of 1:1000 diluted pararosaniline hexasonium salt for 2–3 hrs at 20° C. Hexosaminidase-containing cells stain increasingly pink-red in direct proportion to their level of enzyme activity<sup>30</sup>. NASBG staining of primary neural cells from dissociated brains of wildtype mice served as a positive control for both the intensity of normal staining (identical to that in FIGS. 3B,C) and the percentage of NASBG+cells (~100%). Neural cell types in the primary dissociated cultures were identified by ICC for standard neural cell type-specific markers: for neurons, NeuN (1:100); for astrocytes, GFAP (1:500); for oligodendrocytes, CNPase (1:500); for immature, undifferentiated neuroepithelial-derived progenitors, nestin (1:1000). The presence of the  $\alpha$ -subunit of human  $\beta$ -hexosaminidase was detected via ICC with a specific antibody<sup>30</sup>. Cells were assessed for dual immunoreactivity to that antibody and to the neural cell type-specific antibodies in order to assess which types of mutant Tay-Sachs CNS cells had internalized the cross-corrective enzyme from the human NSCs. Intracytoplasmic GM<sub>2</sub> ganglioside was also recognized by a specific antibody<sup>39</sup>.

Transplantation. NSCs, dissociated as above, were resuspended at  $4 \times 10^4$  cells/ $\mu$ l in phosphate buffered saline (PBS) +0.045% trypan blue and maintained well-triturated on ice until transplanted. For some transplant paradigms, the lateral cerebral ventricles of cryoanesthetized postnatal day 0 (P0) mice were visualized by transillumination, and a 2 $\mu$ l cell suspension was injected gently into each ventricle via a pulled glass micropipette as previously described<sup>29,30</sup>. For EGL transplants, the cerebella of P0 mice were transilluminated and a 2 $\mu$ l cellular suspension was similarly implanted directly into the EGL of each cerebellar hemisphere and the vermis as previously described<sup>13,31</sup>. These animals were sacrificed after the completion of cerebellar organogenesis. All transplant recipients and untransplanted controls received daily cyclosporin (10mg/kg IP) (Sandoz) beginning on the day of transplant. Detection and Characterization of Donor Human NSCs In Vivo. Transplanted mice were sacrificed and perfused with 4% PFA (in 0.1M PIPES buffer, pH 6.9) at serial time points: P1, P2, and weekly through 5 weeks of age. Brains were cryosectioned at 20 $\mu$ m intervals. Donor-derived cells were recognized in a number of ways. Cells which had been transduced with lacZ were identified by either Xgal histochemistry or by ICC with an anti- $\beta$ gal antibody as detailed below<sup>13,32</sup>. All NSCs (even those carrying lacZ) were pre-labeled prior to transplantation with BrdU or with the non-diffusible vital red fluorescent membrane dye PHK-26 (Sigma). For BrdU labeling, 20 $\mu$ M of the



nucleotide analog was added to cultures 48 hrs prior to transplantation; these cells were subsequently identified *in vivo* by an anti-BrdU antibody as described below. PHK-labeled cells, prepared as per the manufacturer's instructions, were detected by fluorescence through a Texas Red (TR) filter. The presence of human donor-derived cells in mouse hosts and the confirmation of the human origin of specific neural cell types *in vivo* was also confirmed through the use of human-specific anti-neural cell type antibodies and through FISH using a probe specific for the human chromosomal centromere. Both procedures are described below. To visualize cellular nuclei of both host and donor cells and to help confirm anatomical location and relationships, some sections were also incubated (for 10 min at 20° C.) in the blue fluorescent nuclear label DAPI.

ICC. ICC with human-specific anti-neural cell type antibodies was performed using standard procedures. Cryosections from engrafted brains were permeabilized in 0.3% Triton X-100 and incubated overnight at 4° C. with an anti-human-NF antibody (1:150; Boehringer) or with an anti-human-GFAP antibody (1:200; Sternberger). Immunoreactivity was revealed by a fluorescein-conjugated anti-mouse IgG secondary antibody (1:200; Vector). Sections were also incubated in DAPI to visualize cellular nuclei. When donor-derived cells were to be recognized by the presence of Bgal or BrdU, then the phenotypes of these cells were characterized by incubating tissue sections with antibodies to either Bgal or BrdU in conjunction with antibodies to neural cell type-specific markers. Sections for Bgal immunostaining (1:1000; Capel) were prepared as above. To reveal BrdU-intercalated cells via ICC, tissue sections were prepared somewhat differently. They were rehydrated in PBS, incubated in 2N HCl at 37° C. for 30 min, washed twice in 0.1M sodium borate buffer (pH 8.3), washed thrice in PBS, permeabilized in 0.3% Triton X-100, and incubated with an anti-BrdU antibody (1:10; Boehringer) at 37° C. for 1 hr. Immunoreactivity was revealed with either a fluorescein-conjugated (1:250; Jackson) or a biotinylated (1:200; Vector) secondary antibody. The neural cell type-specific antibodies employed were as follows: anti-NF (1:250; Sternberger) and anti-NeuN (1:20; gift of R. Mullen) to identify neurons; anti-CNPase (1:200–1:500; Sternberger) to identify oligodendrocytes; anti-GFAP (1:150; Sigma) to identify astrocytes. Immunostaining for these markers was revealed with a TR-conjugated secondary antibody [1:200; Vector]. Vmyc expression (unique to donor-derived cells) was assessed with an antibody to the protein (1:1000; UBI).

FISH for the Human-Specific Centromere. Animals were perfused with 4% PFA+2% glutaraldehyde. Cryosections of brain were post-fixed in 4% PFA, permeabilized in 0.2% Triton X-100, washed in PBS, incubated in 0.2N HCl for 20 mins., washed again in PBS, and then exposed to proteinase K (100 µg/ml) in 0.1M Tris, 0.005M EDTA (pH 8.0) at 37° C. for 15 mins. The sections were then washed in 0.1% glycine in PBS for 2 mins. and then in PBS for 5 mins. Sections were post-fixed again in 4% PFA, washed in PBS, and then rinsed with 50% formamide, 2xSSC for 5 mins. A digoxigenin-labeled probe, complementary to regions of the centromere present uniquely and specifically on all human chromosomes (Oncor), was then added to the sections which were coverslipped, sealed with rubber cement, denatured at 100° C. for 10 mins., and placed on ice for 5 mins. Sections were hybridized for 15 hrs at 37° C., following which they were washed in 65% formamide, then 2xSSC for 30 mins. at 43° C., and then 2xSSC for 30 mins. at 37° C., and finally PBS for 15 mins. at 20° C. The probe was detected by an

anti-digoxigenin TR-conjugated antibody (Boehringer) diluted 1:5 in 0.5% BSA +5% NHS in PBS (30 mins. at 37° C.). Slides were rinsed 3 times in PBS (15 mins./wash), incubated with DAPI, rinsed again in PBS, and mounted for fluorescence microscopy.

## RESULTS

### Isolation, Propagation, and Cloning of Human Neural Stem Cells.

The isolation, propagation, characterization, cloning, and transplantation of NSCs from the human CNS followed a "blueprint" propagated following transduction of a constitutively downregulated vmyc and growth factor- (especially bFGF-) expanded NSC clones. Based on the observation that NSCs—even genetically propagated clones<sup>34</sup>—appear to divide in response to bFGF and EGF in serum-free medium<sup>5,35</sup>, this dual responsiveness was chosen as a method for both screening and for enriching a starting population of dissociated primary human neural tissue for cells that fulfilled this requirement. In addition, the primary neural cells were initially obtained (to the best of our ability) from a region of the CNS postulated to harbor (in lower mammals) a relatively rich population of NSCs, the ventricular zone (VZ) of the fetal telencephalon<sup>1,7,8,23</sup>.

Dissociated neural cells from a 15 week human fetal brain were initially grown as a polyclonal population in serum-free medium containing bFGF and/or EGF. The cultured cells were often transferred between media containing one or the other of the two mitogens in order to establish and select for dual responsiveness. Some populations were then maintained in bFGF alone for subsequent manipulation and cloning as described below; others were used for retrovirally-mediated transduction of vmyc and subsequent cloning, also as described below.

In order to provide an unambiguous molecular tag for assessing the clonal relationships of the cells, as well as to facilitate identification of some cells following transplantation and to assess their capacity to express exogenous genes *in vivo*, some bFGF-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding both lacZ (the gene for *E. coli* β-galactosidase [Bgal]) and neo (the gene for neomycin resistance). Infected cells were selected for survival in G418 (a neomycin analog). Single colonies were initially isolated by limiting dilution; monoclonality of the cells in a given colony was then confirmed by demonstrating that only one copy of the lacZ/neo-encoding retrovirus, with a unique chromosomal insertion site, was present. Genomic DNA from individual colonies, digested with restriction endonucleases that cleaved once in the integrated provirus, were subjected to Southern analysis with a radiolabeled probe complementary to neo. In clone Hi, for example, all lacZneo+ cells, indeed, had a single, common retroviral integration site indicating that they were derived from a single infected "parent." Some populations enriched for human NSCs as described above, were transduced with vmyc, yielding multiple stable clones. Specifically, bFGF-maintained cells were infected with an amphotropic, replication-incompetent retroviral vector encoding vmyc and neo<sup>28</sup>. Individual, G418-resistant colonies were isolated and maintained as separate cultures. Again, monoclonality of each colony was affirmed by demonstrating that each putative clone had only one unique retroviral insertion site [FIG. 1B]. All vmyc-transduced human cell colonies (as well as the established murine NSC clone C17.2, included as a positive control) produced single bands of the same molecular weight following endonuclease bisection of the provirus and hybridization to a vmyc probe. Thus, as was the



case for the epigenetically-maintained cells, all the vmyc transduced colonies were monoclonal. Five clones (H6, H9, D10, C2, E11) were generated by this procedure and were maintained in serum-free medium containing bFGF.

To be classified as a NSC, a given cell must yield progeny in all neural lineages and give rise to other single cells that can do so; therefore, it was imperative to affirm unambiguously that all cells in a given colony were members of the same clone. Having met this obligate criterion, additional characterization of clones could proceed.

#### In Vitro Characterization of Clones Multipotency and Self-Renewal

In uncoated dishes and in serum-free medium supplemented with bFGF, all clones grew in culture as clusters that could be passaged weekly for at least 1 year [FIG. 2A]. The cells within these clusters expressed the intermediate filament vimentin, a frequently used and reliable marker for immature multipotent neural progenitors<sup>25</sup>. By dissociating these clusters and plating them in serum-containing medium, these clones differentiated spontaneously into neurons and oligodendrocytes [FIGS. 2B,D], a technique previously used for differentiating bFGF-propagated rodent NSCs and progenitors<sup>16,25,37</sup>. After 5 days under these differentiating conditions, a majority of the cells (90%) in all clones became immunoreactive for the neuronal marker neurofilament (NF) [FIG. 2B], while a smaller proportion (10%) expressed CNPase, a marker for oligodendroglia [FIG. 2C]. Mature astrocytes expressing glial fibrillary acidic protein (GFAP) were not initially appreciated under these culture conditions, even after one month *in vitro*. However, GFAP expression could be induced in these human clones by co-culture with primary dissociated embryonic murine CNS tissue [FIG. 2D]. In addition to cells expressing the variety of differentiated lineage-specific markers noted above (establishing "multipotency"), each clone also gave rise to new immature vimentin+ cells [FIG. 2E] which could, in turn, on subsequent passages, give rise to new cells expressing multiple differentiated neural markers as well as to new vimentin+passageable cells (i.e., "self-renewability").

These results affirmed that all of the clones to be studied *in vitro* and *in vivo* following transplantation fulfilled the operational definition of NSCs: stable, self-renewing, multipotent vimentin+ clones derived from a single cell with the capacity for differentiating into progeny in all 3 fundamental neural lineages. All the clones, whether genetically-modified or epigenetically-maintained, were remarkably similar in their behaviors and phenotypes *in vitro*. To continue this comparison, these various clones were used in parallel in the *in vivo* transplant experiments described below.

#### Storage

Of note, all clones could be efficiently cryopreserved (detailed under Experimental Protocols) with minimal adverse effects on cell viability and no discernible effect on proliferation or differentiation upon thawing.

#### Ability to Complement and Cross-Correct a Genetic Defect

To help assess their potential as vehicles for molecular therapies, we next evaluated, under well-controlled and observable conditions, the ability of human NSCs to complement and cross-correct a prototypical genetic, metabolic defect with the same efficiency as murine NSCs<sup>30,29,38</sup>. The index genetic defect chosen was the absence of the  $\alpha$ -subunit of  $\beta$ -hexosaminidase, the mutation responsible in humans for the untreatable and inexorably neurodegenerative lysosomal storage disease, Tay-Sachs. Absence of the  $\alpha$ -subunit leads to a deficiency in hexosaminidase A which is required to metabolize GM<sub>2</sub> ganglioside to GM<sub>3</sub>. Pathologic

GM<sub>2</sub> accumulation in the brain leads to the progressive neurodegeneration characteristic of the disease. The ability of human NSCs to effect cross-correction was compared with the following two well-established murine NSCs: "clone C17.2" and a subclone of C17.2 (called "clone C17.2H") engineered via retroviral transduction of the  $\alpha$ -subunit to overexpress hexosaminidase<sup>30</sup>. Previous studies using these murine NSC clones established that they both synthesized and secreted significant amounts of functional hexosaminidase A which could be endocytosed by fibroblasts from human Tay-Sachs patients<sup>30</sup>. The recent generation of a transgenic mouse in which the gene encoding the  $\alpha$ -subunit was specifically mutated<sup>39</sup> now permitted an examination of the capacity of human NSCs (as well as the murine NSCs) to cross-correct actual neural cells derived from an animal model of Tay-Sachs. Furthermore, the availability of established, standardized tools for the rapid and reliable detection of the specific gene product, the lysosomal enzyme activity, and the intracellular pathologic phenotype made this model ideal.

To assess their ability to produce a secretable gene product capable of rescuing a mutant neural phenotype, NSCs (murine and human) and control cells were cultured on one side of a membrane with a pore size (0.4  $\mu$ m) sufficient to allow passage of hexosaminidase but not of the cells. The membrane was partially immersed in a well on the bottom of which rested a coverslip onto which neural cells dissociated from the brain of a neonatal Tay-Sachs mouse had been plated. After 10 days of co-culture in this transwell system, the coverslips bearing the mutant neural cells were examined as follows: (1) for the presence of hexosaminidase activity, as assayed by the production of a red precipitate following exposure to naphthol-AS-BI-N-acetyl- $\beta$ -D-glucuronide (NASBG) [FIGS. 3A-C,M]; (2) with both an antibody to the  $\alpha$ -subunit and antibodies to CNS cell type-specific markers to determine which Tay-Sachs neural cell types had internalized the corrective gene product [FIGS. 3D-L]; and (3) for reduction in GM<sub>2</sub> storage [FIG. 3N].

While there was minimal intrinsic hexosaminidase activity in Tay-Sachs CNS cells cultured alone [FIG. 3A], hexosaminidase activity increased to normal intensity when the cells were co-cultured with murine or human NSCs [FIGS. 3B,C]. The extent of human NSC-mediated cross-correction was as successful and efficient as that effected by murine NSCs, yielding percentages of NASBG+Tay-Sachs CNS cells significantly greater than in untreated controls ( $p < 0.01$ ) [FIG. 3M]. As suggested by FIGS. 3D-L, all neural cell types from the Tay-Sachs mouse brain (neurons, glia, progenitors) were corrected. As an indication that this increased enzyme activity favorably impacted the fundamental cellular neuropathologic process, the percentage of Tay-Sachs CNS cells without abnormal intracytoplasmic GM<sub>2</sub> accumulation was significantly lower in those exposed to secretory products from human NSCs than in untreated Tay-Sachs cultures ( $p < 0.01$ ), approaching those from wild-type mouse brain [FIG. 3N].

Therefore, human NSC clones are capable of producing and secreting a functional gene product with sufficient efficiency to be utilized by targeted impaired CNS cells of multiple lineages to correct a genetically-based defect and reduce pathology. These data help establish their validity as potential vehicles for gene therapy.

#### In Vivo Characterization of Clones.

#### Multipotency, Plasticity, & Pursuit of Developmental Programs *In Vivo*

We next determined whether human NSC clones (whether epigenetically or genetically-propagated) could not only

engraft following transplantation into the immature brain, but could also respond appropriately and accommodate to normal developmental cues *in vivo*: migrating appropriately, integrating into host parenchyma, differentiating into neural cell types appropriate to a given region at a given developmental temporal "window"—even if that window differed from when the NSCs were obtained. Dissociated cells from the individual human NSC clones were injected bilaterally into the lateral ventricles of newborn mice, allowing them access to the SVZ. Prior to transplantation, some human cells were transduced, as detailed above, with the lacZ reporter gene. To control for and circumvent the risk of transgene downregulation (with the attendant risk of missed identification of engrafted, donor-derived cells), cells were also prelabeled either by *in vitro* exposure to BrdU 2 days prior to transplantation and/or with the non-diffusible vital fluorescent membrane dye PHK-26 immediately before transplantation. Stably engrafted cells were then detected, as appropriate, by Xgal histochemistry; by immunocytochemistry (ICC) with antibodies directed against BrdU,  $\beta$ gal, human-specific NF and/or human-specific GFAP; by fluorescence *in situ* hybridization (FISH) using a human-specific pan-chromosomal  $\alpha$ -centromeric probe; and/or by PHK fluorescence. For some cells, multiple identification techniques were employed to confirm their donor, human-origin. Cell type identity of donor-derived cells was also established as necessary by dual staining with antibodies directed against neural cell type-specific markers.

Following intraventricular implantation, human NSC clones displayed developmentally-appropriate behavior, emulating that of their murine counterparts [FIGS. 4,5]. Furthermore, the engraftment, migration, and differentiation of epigenetically-perpetuated clones was identical to that of genetically-(vmyc)-perpetuated clones. (free of the 5 clones in the latter category engrafted well [Table]; the remaining 2 clones which engrafted poorly will be discussed below). Both epigenetically- and genetically-maintained donor cells integrated into the SVZ within 48 hours following implantation [FIGS. 4A,B; 5A,K-N]. It is known that endogenous, host progenitors derived from the SVZ migrate out dorsally and laterally into the subcortical white matter and cortex over a 28 day period and become astroglia and oligodendroglia; gliogenesis predominates in those regions at that stage while neurogenesis has ceased<sup>44</sup>. In an analogous manner, engrafted human NSCs also migrated out extensively along the subcortical white matter by 2 weeks following engraftment [FIG. 4C], and, by 3-5 weeks, had appropriately differentiated into oligodendrocytes and astrocytes [FIGS. 5H-J]. Interestingly, the ready detection of donor-derived astrocytes *in vivo* [FIG. 5J] contrasts with the initial absence of mature astrocytes when human NSC clones were maintained *in vitro* in isolation from the *in vivo* environment (recall that co-culture with primary mouse CNS was required to induce astrogliogenesis [FIG. 2D]). Instructive signals emanating from other components of the murine CNS appear necessary for triggering or promoting astrocytic differentiation and/or maturation from multipotent cells.

The second known fate of endogenous SVZ progenitors is to migrate anteriorly along the RMS and differentiate into OB interneurons. By one week following transplantation, a subpopulation of donor-derived human cells from the SVZ were noted migrating along the RMS [FIGS. 4D,E]. In some cases, these cells in the RMS migrated together in small groups [FIG. 4E], a behavior typical for endogenous murine SVZ precursors<sup>43,44</sup>. Three weeks following transplantation, as per the expected fate of progenitors within the RMS, a

subpopulation of donor-derived neurons of human origin (e.g., human-specific-NF+cells) were present within the parenchyma of the OB, intermingled with host neurons [FIGS. 5B-G, which represent high power views of sections through the OB as pictured in FIG. 4D]. Not only were these donor-derived cells human-NF+ [FIG. 5D], but, when sections through the OB were reacted with both an antibody against BrdU (to identify pre-labeled donor-derived human cells) and with an antibody to the mature neuronal marker NeuN, a large number of double-labeled BrdU+/NeuN+ donor-derived cells of human origin were integrated within the granule layer [FIGS. 5E-G], mimicking the NeuN expression pattern of endogenous, host, murine interneurons [FIGS. 5F,G].

To examine further the degree of plasticity of human NSCs, identical clones were implanted into a different germinal zone at the opposite end of the neuraxis. Transplants of the same human NSCs into the EGLs of newborn mouse cerebella appropriately yielded different neuronal cell types in this different location, primarily cerebellar granule cell interneurons in the IGL [FIGS. 6C-I], a result discussed in greater detail in the next section.

Therefore, *in vivo*, as *in vitro* [FIG. 2], all engraftable human NSC clones can give rise to cells in all 3 fundamental neural lineages—neurons [e.g., FIGS. 5D-G; 6]; oligodendrocytes [e.g., FIGS. 5H,I]; and astrocytes [e.g., FIG. 5J]. Not only did transplanted brains look histologically normal (donor cells migrated and integrated seamlessly into the host parenchyma yielding no discernible graft margins), but engrafted animals exhibited no behavioral abnormalities or other indications of neurologic dysfunction. Thus brain structures that received contributions from donor human NSCs appeared to have developed normally.

In these transplant studies, while most clones engrafted quite well, 2 of the 5 genetically-propagated NSC clones appeared to engraft poorly [Table]. Nevertheless, *in vitro* these clones did display characteristics seemingly identical to those of the more robustly engrafting clones. Thus, ostensibly equivalent multipotency *in vitro* does not necessarily translate into equivalent potential *in vivo*, suggesting the need for investigators to test each clone individually in transplantation paradigms. This observation further warns that transplantation of mixed polyclonal populations (the starting material for many transplantation studies presently in the literature<sup>16,33,41,45,46</sup>) because of their inevitable changing character and shifting representations of various clones, may be a problematic strategy.

#### Foreign Transgene Expression *In Vivo*

Gene therapy paradigms, especially for diseases characterized by extensive, multifocal, or global lesions, require that donor cells be capable of expressing foreign genes in widely disseminated locations if the clinical situation demands (in addition to being able to integrate in anatomically restricted locations<sup>10,16,32,45,46</sup>). The retrovirally-transduced, lacZ-expressing representative clone pictured in FIGS. 5A-C (following transplantation into the neonatal mouse cerebral ventricle and SVZ) continued to produce readily detectable  $\beta$  gal after migration to and stable integration and maturation within host parenchyma at distant sites in the mature animal.

#### Spontaneous, Constitutive, Downregulation of vmyc Expression

Interestingly and importantly, in the case of genetically-manipulated human NSC clones, the propagating gene product, vmyc, is undetectable in donor human cells beyond 24-48 hours following engraftment [FIG. 5K-Q] despite the fact that the brains of transplant recipients contain numerous

stably-engrafted, healthy, well-differentiated, non-disruptive, donor-derived cells [FIGS. 4; 5A-J,Q; 6]. These observations suggest that vmyc is regulated by the normal developmental mechanisms that downregulate endogenous cellular myc in CNS precursors during mitotic arrest and/or differentiation. The loss of vmyc expression spontaneously and constitutively from stably engrafted NSCs following transplantation is consistent with the invariant absence of CNS tumors derived from implanted vmyc-propagated NSCs, even after several years in vivo<sup>9,13,29-32</sup>. With human NSCs, brain tumors are never seen.

#### Neural Cell Replacement In Vivo

Having established the probable efficacy in vitro and in vivo of human NSC-mediated foreign gene expression as a basis for gene therapy strategies, we next sought to establish whether such cells could also mediate neural cell replacement. Well-established neurologic mouse mutants have classically provided ideal models for testing specific neural cell replacement paradigms. The meander tail (mea) mutant is one such model of neurodegeneration and impaired development. Mea is characterized by a cell-autonomous failure of granule neurons to develop and/or survive in the cerebellum, especially in the anterior lobe<sup>47</sup>. Murine NSCs are capable of reconstituting the granule neuron-deficient IGL<sup>31</sup>.

In order to assess their ability to replace neurons in-CNS disorders, human NSC clones were engrafted into the EGLs of newborn mea mouse cerebella. When analyzed at the completion of cerebellar organogenesis, donor-derived human cells were present throughout the IGL of the cerebella [FIG. 6]. They possessed the definitive size, morphology, and location of cerebellar granule neurons [FIGS. 6E-G], identical to the few residual endogenous murine host granule neurons with which they were intermixed [FIG. 6G]. That these "replacement" neurons were of human-origin was confirmed by FISH, demonstrating the presence of cells positive for the above-described human-specific chromosomal probe [FIG. 6J]. That the human-derived cells were, indeed, neuronal was confirmed by demonstrating that most engrafted cells in the mea IGL were immunoreactive for NeuN [FIGS. 6H,I], as in the OB, endogenous interneurons in the IGL similarly express NeuN. Thus engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination,

CLONE	PROPAGATION TECHNIQUE	ENGRAFTABLE
H1	bFGF	+
H6	vmyc	+
H9	vmyc	+
C2	vmyc	+
D10	vmyc	-
E11	vmyc	-

\* A neural stem cell is defined as a single cell which is capable of giving rise (in vitro and/or in vivo) to neurons (of multiple types), oligodendrocytes, and astrocytes, as well as new neural stem cells. That these progeny are clonally-related to that single cell and to each other is an obligatory part of the definition. Therefore, an unambiguous demonstration of monoclonality is necessary to affirm both multipotency and self-renewal.

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What we claim is:

1. The living progeny cells of a genetically modified human neural stem cell, wherein said living progeny cells are maintained in-vitro as a stable cell line and suitable for on-demand implantation in-vivo into a living host subject, said living progeny cells comprising:

multipotent descendant cells of human neural stem cell origin which

- (i) remain undifferentiated while maintained in-vitro as mitotic cells;
- (ii) are implantable in-vivo at a chosen implantation site as undifferentiated cells;
- (iii) optionally migrate in-vivo after implantation from the implantation site to another anatomic site for integration within the nervous system of the living host subject;
- (iv) integrate in-situ after implantation into the parenchymal tissues at a local anatomic site in the living host subject; and
- (v) differentiate in-situ after integration into at least one fundamental cell type selected from the group consisting of neurons, oligodendroglia, and astroglia; and

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human genomic DNA genetically modified to include a viral vector carrying at least one DNA segment comprised of an exogenous gene coding for a specific protein.

2. The living progeny cells of a genetically modified human neural stem cell as recited in claim 1 wherein said viral vector is an amphotrophic retroviral viral vector.

3. The living progeny cells of a genetically modified human neural stem cell as recited in claim 1 wherein said viral vector carries an exogenous vmc DNA sequence wherein

- (i) said vmc DNA sequence is highly expressed in-vitro causing said multipotent progeny cells to be in a proliferative cell cycle and rapidly proliferate while maintained in-vitro; and
- (ii) the expression of said vmc DNA sequence becomes controlled and is down-regulated after implantation in-vivo by the constituent tissues of the living host subject such that the rate of in-vivo multipotent progeny cell proliferation markedly decreases.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 6,528,306 B1  
DATED : March 4, 2003  
INVENTOR(S) : Evan Y. Snyder, John H. Wolfe and Seung U. Kim

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

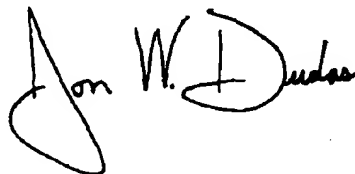
Title page,

Item [73]; Assignees, should read:

-- **The Children's Medical Center Corporation**, Boston, MA (US); **The University of British Columbia** (CA); **The University of Pennsylvania**, PA (US) --

Signed and Sealed this

Sixteenth Day of November, 2004

A handwritten signature in black ink, appearing to read "Jon W. Dudas". The signature is stylized with a large, looping initial "J" and a distinct "D" at the end.

JON W. DUDAS  
*Director of the United States Patent and Trademark Office*

## Direct derivation of conditionally immortal cell lines from an *H-2K<sup>b</sup>*-tsA58 transgenic mouse

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Communicated by Avron Mitchison, January 24, 1991

**ABSTRACT** Studies on cell lines have greatly improved our understanding of many important biological questions. Generation of cell lines is facilitated by the introduction of immortalizing oncogenes into cell types of interest. One gene known to immortalize many different cell types *in vitro* encodes the simian virus 40 (SV40) large tumor (T) antigen (TAG). To circumvent the need for gene insertion *in vitro* to generate cell lines, we created transgenic mice harboring the SV40 TAG gene. Since previous studies have shown that TAG expression in transgenic mice is associated with tumorigenesis and aberrant development, we utilized a thermolabile TAG [from a SV40 strain, tsA58, temperature sensitive (ts) for transformation] to reduce the levels of functional TAG present *in vivo*. To direct expression to a broad range of tissues, we used the mouse major histocompatibility complex *H-2K<sup>b</sup>* promoter, which is both widely active and can be further induced by interferons. tsA58 TAG mRNA was expressed in tissues of all animals harboring the hybrid construct. Development of all tissues was macroscopically normal except for thymus, which consistently showed hyperplasia. Fibroblast and cytokeratin<sup>+</sup> thymic epithelial cultures from these mice were readily established without undergoing crisis and were conditionally immortal in their growth; the degree of conditionality was correlated with the levels of tsA58 TAG detected. One strain of *H-2K<sup>b</sup>*-tsA58 mice has been bred through several generations to homozygosity and transmits a functional copy of the transgene.

Although the use of cell lines has been of central importance in the development of cellular and molecular biology, the limited number of available cell lines and the difficulty in obtaining new ones have impeded many areas of study. The increasing realization of the value of cell lines has been associated with a continual evolution in relevant technologies. Initially, cell lines were obtained only as tumor cells or as spontaneously immortalized variants of cells that grew readily in tissue culture (1). More recently, transfection and retroviral-mediated gene insertion of immortalizing genes have been used to facilitate the production of cell lines from various tissues (2–11). However, transfection requires a large number of target cells to ensure that some cells of interest stably integrate the chosen DNA in a position suitable for expression. Viral-mediated gene transfer can be carried out with fewer cells by cocultivation of target cells with virus-producing feeder layers; however, this method still requires that target cells are dividing to achieve integration of the selected DNA into the genome (2). Moreover, both of these technologies require the growth of cells for extended periods of time in culture, under selective pressure, to obtain sufficient numbers of cells expressing the immortalizing gene to allow experimentation. In addition, lines from putatively

identical cells have different sites of gene integration and often express markedly different behaviors and levels of expression of the immortalizing gene.

An additional problem associated with the introduction of immortalizing genes into cells is that these genes can alter normal cellular physiology (1, 12), a problem that is also relevant to the isolation of cell lines from transgenic animals (e.g., refs. 28–30, 34, 35). This problem theoretically can be overcome through the use of conditional immortalizing genes, which allow the generation of continuously proliferating cell lines capable of differentiation after inactivation of the immortalizing gene. For example, the simian virus 40 (SV40) mutant temperature-sensitive (ts) strain tsA58, which encodes a thermolabile large tumor (T) antigen (TAG) capable of immortalization only at the permissive temperatures, has been used in the generation of a variety of conditionally immortal cell lines (13–17). However, introduction of conditional immortalizing genes *in vitro* still suffers from the problems discussed above for transfection and infection of wild-type genes.

To overcome some of the difficulties in the generation of cell lines, an approach was developed that facilitates and ensures the presence of a conditional oncogene in all of the cells of interest at a common integration site. Thus, transgenic mice were generated that harbor SV40 strain tsA58 early region coding sequences under the control of the mouse major histocompatibility complex *H-2K<sup>b</sup>* class I promoter (18–21). This promoter is active at various levels in different tissues of the body but can be induced to higher levels of expression in almost all cells by exposure of the cells to interferons (IFNs) (21–23). Skin fibroblast cultures derived from these mice were conditional in their growth, as has been demonstrated for rat embryo fibroblasts immortalized by infection with a recombinant retrovirus that transduces the tsA58 TAG (15). Work with transfection and viral-mediated gene insertion has consistently indicated that techniques developed through the use of fibroblast populations can be transferred readily to other cell systems. This is also the case with the cells obtained from these transgenic mice, and cytokeratin<sup>+</sup> thymic epithelial cell lines that were also established readily from these animals.

### MATERIALS AND METHODS

**Construction of the Transgene.** The 5' flanking promoter sequences and the transcriptional initiation site of the mouse *H-2K<sup>b</sup>* class I gene were fused to the SV40 tsA58 early region coding sequences. The 4.2-kilobase (kb) *EcoRI*–*Nru I* fragment encompassing the *H-2K<sup>b</sup>* promoter sequences was ligated to the 2.7-kb *Bgl I*–*BamHI* fragment derived from the tsA58 early region gene and pUC19 double-digested with

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Abbreviations: TAG, large tumor (T) antigen; ts, temperature sensitive; IFN, interferon; mAb, monoclonal antibody; SV40, simian virus 40.

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*EcoRI* and *BamHI*. The *Bgl* I site was blunted by using the Klenow fragment of *Escherichia coli* DNA polymerase I to allow fusion to the *Nru* I site. For microinjection, the *H-2K<sup>b</sup>-tsA58* DNA fragment was isolated free of vector sequences by digestion with *EcoRI* and *Sal* I (24). All DNA manipulations were carried out by standard procedures (25).

**RNA Blot-Hybridization (Northern) Analysis.** RNA was prepared and analyzed by hybridization to a <sup>32</sup>P-labeled SV40 early region fragment using standard procedures (25, 26).

**Cloning and Proliferation Assays.** Skin fibroblasts were prepared as described (27) and grown in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin, streptomycin, and recombinant murine  $\gamma$  interferon (IFN- $\gamma$ , Genzyme) per ml. For colony assays, 10<sup>3</sup> cells derived from cultures grown at 33°C in the presence of IFN- $\gamma$  were replated in 6-cm tissue culture dishes in the absence of IFN- $\gamma$  at 33°C to allow adherence under identical conditions. Growth conditions were changed after 24 hr to indicated conditions (Fig. 2). Cultures were refed twice weekly for 14 days and stained with 2% methylene blue; colonies then were counted blind. For proliferation assays, 10<sup>4</sup> cells were similarly plated, and dishes were analyzed after 7 and 14 days. A single dish was also counted on day 1 to determine the number of adhering cells. All determinations were carried out in duplicate.

**Immunoblot (Western Blot) Analysis.** Preparation of protein extracts and their analysis with mAb PAb419, directed against TAG, were performed by standard procedures (26).

**Immunofluorescence Analysis of Thymic Epithelial Cells.** Cells grown on poly(L-lysine)-coated coverslips were stained with an antibody specific for keratin 8 (LE41; ref. 32) or an anti-TAG mAb, PAb412 (31).

## RESULTS

**Generation of *H-2K<sup>b</sup>-tsA58* Transgenic Mice.** A hybrid construct containing the *H-2K<sup>b</sup>* 5' promoter sequences fused to the tsA58 early region gene, which encodes both TAG and the small tumor antigen (Fig. 1 Upper), was microinjected into fertilized oocytes from (CBA/Ca  $\times$  C57BL/10) F<sub>1</sub> mice. After reimplantation, 88 mice were born, of which 34 carried one to five copies of the gene. RNA from a variety of tissues from one nontransgenic and three transgenic animals was analyzed by Northern blot analysis with an SV40 early region-specific probe (Fig. 1 Lower). RNA extracted from tissues of transgenic mice contained various amounts of a 2.5-kb RNA species, while no tsA58 TAG RNA was detected in tissues of the nontransgenic mouse; thymus and liver showed the highest level of expression, while brain showed the lowest.

**Fibroblasts Derived from *H-2K<sup>b</sup>-tsA58* Transgenic Mice Are Conditionally Immortal.** Skin fibroblasts from normal and founder transgenic animals 2–10 weeks old were placed in culture at 33°C, the permissive temperature for the tsA58 TAG, in the presence of IFN- $\gamma$  (to increase expression from the *H-2K<sup>b</sup>* promoter; refs. 21–23). Fibroblasts derived from nontransgenic mice stopped dividing *in vitro* within a small number of passages. This cessation of division, which has been termed both "senescence" and "crisis," occurs reproducibly in fibroblasts that do not express immortalizing genes. In contrast, fibroblasts derived from most transgenic mice continued to grow for as long as the cultures were maintained under appropriate conditions (see below).

Detailed analysis of skin fibroblast cultures for conditionality of growth revealed three families of cultures, depending upon the ability of cells to grow in fully permissive, semipermissive, and nonpermissive conditions. Permissive conditions were defined as growth at 33°C in the presence of IFN- $\gamma$ ; semipermissive conditions, growth at 33°C in the absence of IFN- $\gamma$  or 39.5°C in the presence of IFN- $\gamma$ ; and

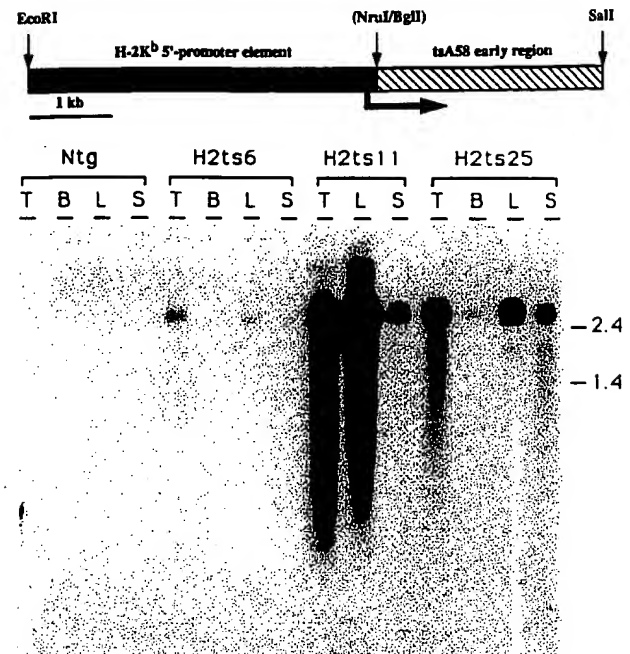


FIG. 1. (Upper) Schematic representation of the *H-2K<sup>b</sup>-tsA58* fragment. Size in kb is indicated. (Lower) Northern blot analysis shows TAG mRNA at various levels in thymus (lanes T), brain (lanes B), liver (lanes L), and skin (lanes S) of different *H-2K<sup>b</sup>-tsA58* transgenic mice. Loading of RNA was checked by hybridization of the same filter with an actin probe (not shown). Size in kb is indicated on the right. Ntg, nontransgenic.

nonpermissive conditions, growth at 39.5°C in the absence of IFN- $\gamma$  (Fig. 2).

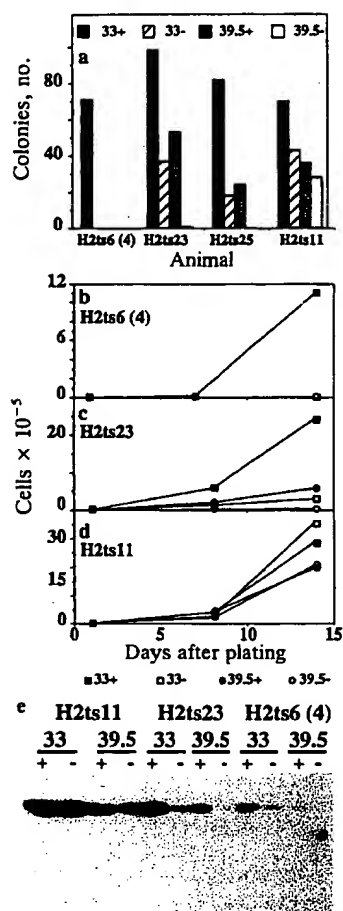
In the first family of cultures, growth was fully conditional and only occurred under permissive conditions. If cells were grown at 39.5°C and/or were grown in the absence of IFN- $\gamma$ , cell division did not occur either in standard growth assays or in colony-forming assays (Fig. 2). These fibroblasts thus behaved as expected from previous studies in which rat embryo fibroblasts were conditionally immortalized with tsA58 TAG by retroviral infection (15). All cultures derived from different individuals within this strain yielded identical results.

In a second family of cultures, optimal growth was obtained under fully permissive conditions, a lesser degree of growth was seen under semipermissive conditions, and no growth occurred under nonpermissive conditions. In the third family, cell growth did not completely cease even under nonpermissive conditions, although the best growth was seen under fully permissive conditions.

The conditionality of growth seen in fibroblasts derived from transgenic animals was correlated with the levels of tsA58 TAG (Fig. 2e). In all cultures, the level of tsA58 TAG was reduced by temperature increase and/or by removal of IFN- $\gamma$ . Interestingly, when the most conditional cultures (those derived from progeny of mouse H2ts6) were grown at 33°C in the absence of IFN- $\gamma$ , a condition where these cells did not grow, low levels of TAG were still detected (Fig. 2e).

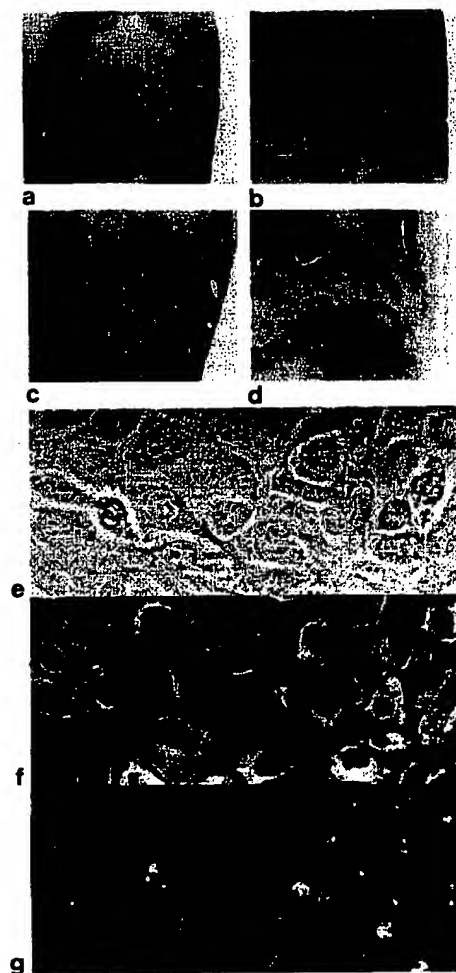
**Thymic Hyperplasia in *H-2K<sup>b</sup>-tsA58* Transgenic Mice.** Enlarged thymuses occurred in all transgenic animals, a tissue-specific hyperplasia that previously has been observed in transgenic mice harboring wild-type TAG (33, 34); the time of onset of hyperplasia (2–20 weeks) was correlated with the levels of TAG mRNA (see Fig. 1 Lower). Despite the thymic enlargement, there was no evidence for malignant transformation of this tissue as judged by the following criteria: both lobes of the thymus were equally enlarged in all animals examined, and histological and immunohistochemical examination revealed





**FIG. 2.** (a-d) Cloning and proliferation analysis of skin fibroblasts from *H-2K<sup>b</sup>-tsA58* transgenic mice reveals three families of cells. In family 1 (mouse H2ts6-4), cloning and proliferation are fully conditional and only occur when cells are grown at 33°C in the presence of IFN-γ (33+) (a and b). In family 2 (mice H2ts23 and H2ts25), optimal results were obtained when cells were grown at 33°C in the presence of IFN-γ (33+), no growth occurred at 39.5°C in the absence of IFN-γ (39.5-), and intermediate levels of growth were seen in the semipermissive conditions of 33°C, IFN-γ<sup>-</sup> (33-) or 39.5°C, IFN-γ<sup>+</sup> (39.5+) (a and c). In family 3 (mouse H2ts11), growth occurred in all conditions but was most vigorous at 33°C in the presence of IFN-γ (a and d). A reduced cloning efficiency and rate of cell growth was seen in semipermissive conditions, and a still greater reduction was seen in fully nonpermissive conditions. (e) Western blot analysis of skin fibroblasts shows that the levels of TAG are correlated with the conditionality of *in vitro* growth. The most conditional cells (derived from progeny of H2ts6) contained the lowest levels of TAG, and the least conditional cells (derived from H2ts11) showed the highest levels of TAG. In all cases, the level of TAG present increased upon addition of IFN-γ to the cultures and decreased upon shift to 39.5°C.

extensive growth of epithelial cells and the presence of apparently normal thymocyte populations, as determined by fluorometric cytometry (not shown). In addition, demarcation between cortical and medullary regions was still maintained even after prolonged hyperplastic growth (Fig. 3). Moreover, dissociated cells obtained from enlarged thymuses did not yield tumors in syngeneic recipients even when  $10^7$  cells were injected s.c. or i.p., and recipient animals were sacrificed after 3 months (unpublished observations). Finally, analysis of T-cell receptor  $\beta$  chain gene rearrangements by Southern blot of DNA from enlarged thymuses suggested polyclonal expansion of thymocyte populations (Y.T., unpublished observations), in contrast to the oligoclonal expansion observed in mice that harbor a hybrid *Thy-1-myc* gene (35). As it is possible that the



**FIG. 3.** (a-d) Histological analysis of thymic tissues from a nontransgenic mouse (a) and from H2ts6 mice 2 months (b), 4 months (c), and 6 months (d) old. The thymuses of H2ts6 mice up to 2 months old appeared to be identical to those of normal mice (a and b), exhibiting normal ratios between cortex (darkly stained tissue) and medulla (lightly stained tissue). The thymic architecture in 4-month-old mice (c) showed signs of disruption, while areas with cortical or medullary characteristics were still maintained. In thymic tissue of 6-month-old H2ts6 mice, extensive lightly staining areas were evident even in subcapsular regions. However, even in these organs a clear demarcation between "cortical" and "medullary" areas was still maintained. (e-g) Immunofluorescent staining of thymic epithelial cells. Cells from an adherent cell line (7P) derived from the thymus of H2ts23 were photographed under: phase optics, showing flattened cells with tightly apposed borders (e); optics with fluorescein isothiocyanate, showing filamentous cytoplasmic staining characteristic of keratins (f); and optics with rhodamine isothiocyanate, indicating the presence and nuclear localization of TAG in virtually all cells (g). ( $\times 200$ .)

large number of highly proliferative and hyperplastic cells in the thymus represents a target in which secondary cooperating mutations might occur, we cannot exclude the possibility that a very small number of cells within the hyperplastic thymus have undergone transformation.

In heterozygous progeny of one mouse (H2ts6), the thymus displayed normal development for extended periods, with the first histological appearance of hyperplasia seen at 4 months (Fig. 3). Homozygote offspring of H2ts6 developed thymic hyperplasia earlier (unpublished observations), in agreement with the view that the time of onset of this abnormality is correlated with TAG levels. Thymic hyperplasia was occasionally seen in conjunction with enlargement of peripheral lymphoid organs (spleen, lymph nodes), but these tissues

maintained their normal histological architecture. Macroscopic evidence of liver abnormalities was seen only in one animal even though levels of transgene expression in the liver were comparable to those in the thymus (Fig. 1 Lower).

**Conditionally Immortal Lines of Cytokeratin<sup>+</sup> Thymic Epithelial Cells.** Thymuses of transgenic mice readily yielded conditionally immortal cultures containing cells of both epithelial and fibroblastic morphologies, both of which could be readily cloned. Clones that exhibited epithelial-like morphologies expressed cytokeratin (Fig. 3). Both cytokeratin<sup>+</sup> and cytokeratin<sup>-</sup> clones showed conditional growth. Cells grew optimally in fully permissive conditions and did not grow in nonpermissive conditions (Fig. 4). Thus, we were able to readily derive conditionally immortal lines of epithelial cells and of fibroblasts from these mice.

**Dose Dependence of Skin Fibroblasts Derived from H2ts6 to IFN- $\gamma$ .** The establishment of a colony of *H-2K<sup>b</sup>-tsA58* transgenic mice has allowed us to begin using these animals to study more detailed aspects of TAG function. In particular, observations that fibroblasts derived from progeny of H2ts6 showed a relatively low level of TAG expression at 33°C in the presence or absence of IFN- $\gamma$  (although expression was clearly higher in the presence of IFN- $\gamma$ ; Fig. 2e) suggested that with this animal it might be possible to observe dramatic alterations in cell growth as a result of small changes in the level of this gene product.

Fibroblasts derived from progeny of H2ts6 mice showed promotion of cell growth by IFN- $\gamma$  at levels as low as 1 unit/ml (Fig. 5). Analysis by colony formation and by cell number analysis showed that addition of IFN- $\gamma$  at 100 units/ml to these cultures only increased the frequency of colony formation 3.5-fold in comparison with that seen in the presence of IFN- $\gamma$  at 1 unit/ml and was only 40% increased over that achieved with IFN- $\gamma$  at 10 units/ml. The difference in TAG levels at the different doses of IFN- $\gamma$  was not large, with 1 unit/ml causing a 2.5-fold increase over basal levels of TAG and 100 units/ml causing an  $\approx$ 6-fold increase over basal levels of TAG.

## DISCUSSION

We have generated transgenic mice that have stably integrated the SV40 mutant strain tsA58 thermolabile TAG gene,

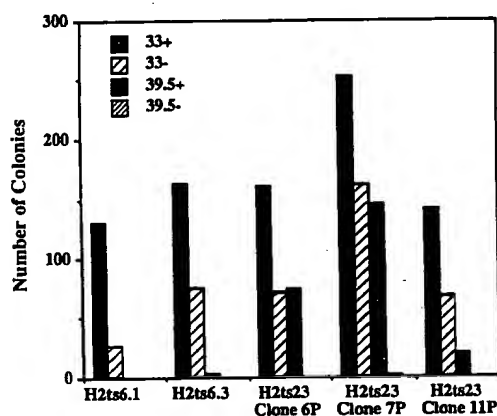


FIG. 4. Analysis of growth of thymic adherent cells by colony formation demonstrates that these cells exhibited conditional growth *in vitro*. Optimal growth occurred under the fully permissive condition of 33°C, IFN- $\gamma$ <sup>+</sup> (33+) in all cases, and no colony formation occurred in nonpermissive conditions. Cultures of cells from H2ts6.1 and H2ts6.3 were derived from the thymuses of two separate progeny of founder mouse 6, and cultures of H2ts23 clones 6P, 7P, and 11P are three separate clonal cultures derived from the thymus cells of animal H2ts23; the clones 6P and 7P were morphologically epithelial, whereas clone 11P was fibroblastic. 33+, 33°C and IFN- $\gamma$ <sup>+</sup>; 33-, 33°C and IFN- $\gamma$ <sup>-</sup>; 39.5+, 39.5°C and IFN- $\gamma$ <sup>+</sup>; 39.5-, 39.5°C and IFN- $\gamma$ <sup>-</sup>.

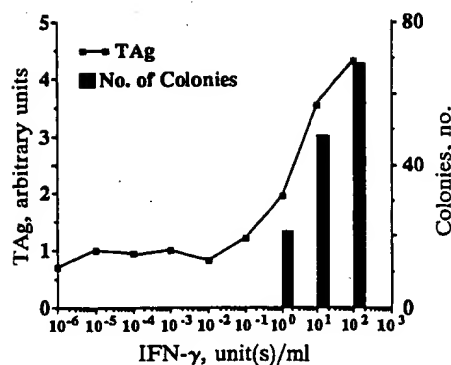


FIG. 5. Maintenance of growth of H2ts6-derived fibroblasts requires low levels of IFN- $\gamma$ . No colonies were obtained in the absence of IFN- $\gamma$ , but the presence of as little as 1 unit of IFN- $\gamma$  per ml was sufficient to allow colony formation. Determination of the level of TAG by Western blot analysis coupled with densitometry showed that the increase in colony formation was associated with no more than a 2.5-fold increase in the level of TAG.

a conditional immortalizing gene, under the control of the inducible 5' flanking promoter of the mouse *H-2K<sup>b</sup>* gene. The tsA58 TAG gene product is functional at the permissive temperature of 33°C but is rapidly degraded at the nonpermissive temperature of 39.5°C (13, 15). The *H-2K<sup>b</sup>* promoter is active in a wide variety of tissues at various levels (18–21), and expression can be increased above basal levels in most cells by exposure to IFN (21–23). Fibroblasts and thymic stromal cells derived from the *H-2K<sup>b</sup>-tsA58* transgenic mice showed conditional proliferation that could be modulated both with temperature and by application of IFN- $\gamma$ ; cells from all mice grew optimally at 33°C in the presence of IFN- $\gamma$ . Founder animal H2ts6, whose progeny yielded fibroblast cultures whose growth in cloning assays was completely dependent upon the permissive temperature and the presence of IFN- $\gamma$ , has bred successfully to homozygosity to yield a strain of *H-2K<sup>b</sup>-tsA58* transgenic mice.

The assay system used to examine conditionality of immortalization was based on results of previous studies in which tsA58 TAG was introduced into fibroblasts by retroviral infection (15). These conditionally immortalized fibroblasts grew indefinitely when maintained at 33°C but rapidly ceased proliferation when switched to 39.5°C. Cells derived from *H-2K<sup>b</sup>-tsA58* transgenic mice behaved similarly. Skin cells from these mice grown *in vitro* at 33°C in the presence of IFN- $\gamma$  readily yielded fibroblast cultures from all transgenic animals. Shift to semipermissive conditions of growth (i.e., 33°C/IFN- $\gamma$ <sup>-</sup> or 39.5°C/IFN- $\gamma$ <sup>+</sup>) was sufficient to eliminate growth of cells derived from the H2ts6 strain of mice. In all other cases, shift to semipermissive conditions was associated with a reduction in cell growth but not a cessation of growth. Cultures from almost all animals ceased growth when shifted to nonpermissive conditions—i.e., 39.5°C, IFN- $\gamma$ <sup>-</sup>. Moreover, in the cultures (family 3 in Fig. 3) in which growth occurred after temperature increase to 39.5°C in the absence of IFN- $\gamma$ , this growth was still less vigorous than that seen in semipermissive conditions. It should be noted that all cultures established from the same founder mouse, or strain of mice, exhibited identical characteristics.

Determination of the amount of TAG present in different cultures by Western blot analysis showed a direct correlation between the amount of TAG present and the growth potential of the cells. Cells in which only small amounts of TAG were produced showed stringent growth regulation, while cultures expressing high levels of TAG showed poor growth regulation. It was also clear that only small increases of TAG were needed to maintain immortalization, in that we saw only a

2.5-fold difference in levels of TAg between untreated cultures and those grown in the presence of IFN- $\gamma$  at 1 unit/ml, yet only the cultures receiving the IFN- $\gamma$  were able to generate colonies in a limiting dilution assay.

Conditional immortalization and the ability to readily generate rapidly growing cultures were also seen with cells derived from thymuses of transgenic mice. As with skin fibroblasts, optimal growth of the thymic cultures occurred at 33°C in the presence of IFN- $\gamma$ , was reduced in semipermissive conditions, and was reduced still further in nonpermissive conditions. Interestingly, thymic cells derived from H2ts6 animals did not grow at 39.5°C in the presence of IFN- $\gamma$  but did grow at 33°C in the absence of IFN- $\gamma$ . This pattern of growth may reflect a higher constitutive level of transcription from the *H-2K<sup>b</sup>* promoter in the thymic cells and/or a greater sensitivity of thymic cells to the action of TAg as compared with fibroblasts. The probable relevance of the first explanation is supported by observations that *in vivo* expression of the transgene in the thymus was generally higher than in other organs, while the relevance of the second explanation is supported by observations that the liver—the one organ in which transgene expression was similar to that of the thymus—rarely showed abnormal growth. The different effects of the transgene on thymus and liver *in vivo* suggest that cell types can differ in their susceptibility to the action of TAg.

Long-term survival of the transgenic mice was correlated with the level of conditionality of growth of the *in vitro* cultures. The only visible cause of physical distress found repeatedly was thymic enlargement. This enlargement seemed to represent hyperplastic growth rather than malignancy because thymic histology, T-cell repertoire, and T-cell clonality were all normal, and cells derived from enlarged thymuses did not generate tumors in syngeneic recipients. Although all populations of the thymus were expanded *in vivo*, only adherent cell cultures were readily obtained in long-term culture, in contrast to cells derived from *Thy-1-myc* mice (35). The generalized hyperplasia of thymic populations we have observed is similar to that seen in transgenic mice when wild-type TAg gene expression was regulated by its own early region promoter (35) or by the promoter from growth hormone-releasing factor gene (34). It differs from the hyperplasia observed in mice where the *H-2K<sup>b</sup>* promoter was used to drive expression of the *fos* oncogene in which expansion of the epithelial component, but not of the lymphoid component, was seen (36).

Although all animals eventually succumbed to thymic hyperplasia, thus indicating that the transgene was not fully inactivated *in vivo*, the H2ts6 heterozygotes survived to the age of 6 months and homozygotes survived to the age of 3 months. Both heterozygotes and homozygotes breed normally in brother/sister matings.

The presence of a viable strain of transgenic mice harboring the *H-2K<sup>b</sup>-tsA58* transgene will allow us to determine whether this approach to cell line production is applicable to tissues—including embryonic tissues—other than skin and thymus. As SV40 TAg can immortalize a wide range of cell types (4, 5, 7, 9, 11, 15–17) and IFN induces the expression of class I genes in a variety of tissues (21–23), the *H-2K<sup>b</sup>-tsA58* transgenic mice may allow direct derivation of cell lines from a wide variety of different tissues and cell types. Moreover, the ability to remove the immortalizing function of the tsA58 TAg in cells derived from these transgenic mice by temperature shift may allow us to generate cell lines that are not only conditional in their growth but also may be capable of differentiating into different types of end-stage cells (see, e.g., refs. 4 and 16). Finally, as cells prepared from these transgenic mice are genetically homogeneous, can be

prepared in large numbers, and can be synchronously exposed to interferon *in vitro*, these cells will allow study of the acute effects of SV40 TAg expression on division and differentiation in the absence of extensive *in vitro* growth and application of drug selection.

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# Immortalization of Precursor Cells from the Mammalian CNS

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## Summary

Recent studies show that the nervous system contains many molecularly distinct cell types. Clonal cell marking experiments demonstrate that different cell types in some areas of the CNS are products of a multipotential stem cell. The factors controlling the differentiation of vertebrate CNS precursor cells would be more accessible to molecular analysis if cell lines with precursor properties could be established. Here we show that cell lines expressing an antigenic marker specific for a major brain precursor cell population can be established from rat cerebellum. We demonstrate that cell lines express the precursor, neuronal or glial properties depending on the growth conditions. This work supports the view that brain precursor cells expressing the marker Rat 401 are multipotential and can differentiate into cells with either neuronal or glial properties. Cell lines capable of differentiation should be useful in defining the signaling systems generating the cell types of the brain.

## Introduction

Brain function requires a large number of distinct cell types. How this cellular diversity is specified is a central question of developmental biology. Lineage analysis of retinal development in flies, frogs, and mice shows that different neuronal and glial cell types can be derived from a multipotential precursor cell (Ready et al., 1978; Lawrence and Green, 1979; Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988). These studies support a model in which the complex cellular organization of the brain is a consequence of interactions between precursor cells and differentiated cells in the neuroepithelium.

To identify the cell types present in the developing mammalian neuroepithelium, we made a set of monoclonal antibodies against embryonic rat nervous system (Hockfield and McKay, 1985). One of these antibodies, Rat 401, identifies a 200 kd protein encoded by a 6.0 kb mRNA that is only expressed in the developing nervous system when neurons are differentiating (Lendahl et al., 1988, Soc. Neurosci. abstract). Rat 401 specifically binds to a large in vivo population of neuroepithelial cells in the

rat with characteristics expected of neuronal and glial precursor cells (Frederiksen and McKay, 1988).

It would be useful to have cell lines with embryonic properties to characterize the signaling pathways in brain precursor cells. In this paper we report that Rat 401-positive cell lines can be established from the developing CNS of the rat. These cell lines are stable, and their state of differentiation can be altered by changing the conditions of growth.

## Results

The feasibility of generating immortal cell lines that express the functions of neuroepithelial precursor cells is influenced by three major factors: the primary cell populations available for immortalization, the immortalizing method, and the conditions and assays used to analyze the putative precursor cells and their differentiated products.

### Proliferating Cerebellar Cell Types

We chose to use the cerebellum as a source of primary cells because neurogenesis in this region is postnatal, allowing easy access to large numbers of cells at early stages of development. As we proposed to use retroviral vectors to establish cell lines from the postnatal cerebellum, we first defined, in vivo, the proliferating cell types that are potential targets for retrovirus-mediated gene transfer. The Rat 401 antigen and vimentin are markers for precursor populations. In addition, a third population may be identified by the expression of the astrocytic marker glial fibrillary acidic protein (GFAP). Dissociated cerebellar cells were stained with Rat 401, anti-vimentin, and anti-GFAP antibodies (Figure 1). The proportion of Rat 401-positive cells is initially large and declines to zero before postnatal day 15 (PN15). These results on dissociated cells are consistent with previous studies on the distribution of rat 401 in sections of the developing cerebellum (Hockfield and McKay, 1985). The proportion of vimentin-positive cells is much larger than the proportion of Rat 401-positive cells, but it also declines over this period. The proportion of GFAP-positive cells is initially lower than the proportion of Rat 401-positive cells, but by the end of the second postnatal week, the number of GFAP-positive cells increases.

Figure 1 shows that these three markers are differentially expressed in cerebellar cell populations, but further data are needed to establish whether these cell populations are independent of one another. The overlap in cell populations was determined by double label immunohistochemistry (Table 1). These results show that the Rat 401-positive population is a subset of the vimentin population. The GFAP-positive population and the vimentin-positive population also overlap.

The proliferative status of the antigenically distinct cell types was measured by an in vivo pulse of [<sup>3</sup>H]thymidine followed by immunohistochemistry and autoradi-

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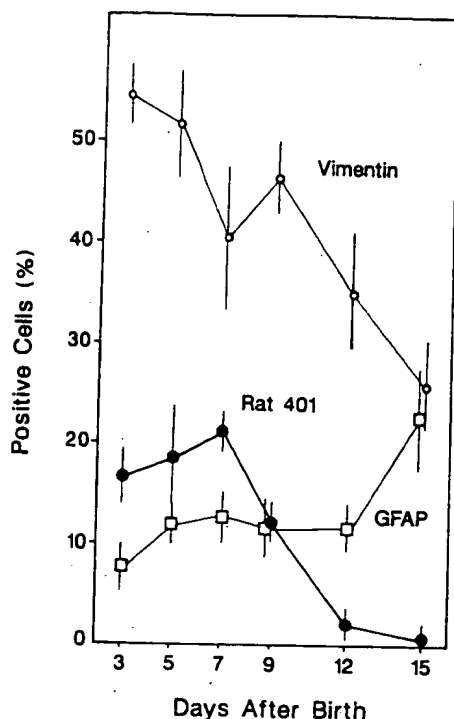


Figure 1. Cell Population Dynamics in the Developing Cerebellum. Dissociated cells prepared from the cerebellum on different postnatal days were stained with anti-vimentin, anti-GFAP, and Rat 401 antibodies. The proportion of labeled cells is expressed as a percentage of total cells. The proportion of Rat 401-labeled cells declines at the end of the period of neurogenesis, and the later period of gliogenesis is reflected in the rising numbers of GFAP-positive cells on PN15.

Table 1. Proportion of Rat 401-Positive and GFAP-Positive Cells That Double-Label with Other Antibodies

Cell Type	% Cell Labeled with Second Antibody		
	Anti-Vimentin	Anti-GFAP	Rat 401
Rat 401-positive	96	53	-
GFAP-positive	80	-	77

ography on dissociated cells (Table 2). It is clear that the three marker antibodies are expressed in proliferating cell populations, suggesting that they may be suitable targets for retrovirus-mediated oncogene transduction.

#### Oncogene Transducing Retroviruses

Three different oncogenes, SV40 T antigen, *v-myc*, and *neu*, were used to establish cell lines from the developing cerebellum. *v-myc* and SV40 T antigen have previously been shown to be efficient in establishing rodent fibroblast cell lines (Land et al., 1983; Jat and Sharp, 1986). In contrast, the *neu* oncogene has not previously been shown to establish primary rodent cell lines. However, activated *neu* is associated with neuroblastomas and glioblastomas (Schechter et al., 1984); in addition, the cellular *neu* is known to be expressed at the transcrip-

Table 2. Proportion of Antibody-Labeled Cell That Are Also Radiolabeled by an In Vivo Pulse of [ $^3$ H]Thymidine 2 hr before Cell Dissociation

Antibody	Percentage of Cell Labeled on Postnatal Day:				
	2	7	9	12	15
Rat 401	8	8	9	-	-
Vimentin	17	16	20	21	25
GFAP	9	11	10	6	5

tional and translational levels in the early nervous system (R. M. and G. Almazan, unpublished data). SV40 has been shown to generate cell lines with either neuronal or glial properties (DeVitre et al., 1974; Neto et al., 1986).

Details of the construction of the *v-myc* transducing retrovirus can be found elsewhere (Dotto et al., 1985). The *neu* transducing retrovirus was a generous gift of Dr. C. I. Bargmann. To explore the differentiation potential of cell lines in the absence of an active oncogene, a transducing retrovirus carrying the tsA58 mutant form of SV40 large T antigen was constructed (Figure 2). SV40 T antigen has the advantage of being available in a "tight" temperature-sensitive form, allowing the function of the oncogene to be switched off by raising the temperature of cell growth (Tegtmeyer, 1975a, 1975b). Holtzer et al. (1975) and Fiszman and Fuchs (1975) showed that cell lines generated with a temperature-sensitive variant of Rous sarcoma virus would differentiate into myotubes when grown at an elevated temperature. Differentiation of a glial cell line carrying a temperature-sensitive oncogene has also been observed (Giotta et al., 1980).

The three recombinant retroviruses used here were all constructed by inserting DNA sequences encoding the oncogenes into the unique BamHI site of the pZip-NeoSV(X)1 vector (Figure 2). The genes were inserted in the sense orientation with respect to retroviral transcription driven by the *cis*-acting transcriptional regulatory sequences in the left-hand long terminal repeat. Previous work has shown that different regulatory sequences allow the same oncogene to interact with different cell types. Because the only differences in the three retroviruses used here were in the coding sequences, any differences in the derived cell lines must be due to the oncogenes themselves and not to the regulatory sequences controlling transcription. All the pZipNeoSVts-A58 producer cell lines were shown to synthesize virus that transduced only large T antigen (see Southern analysis below). Thus the reported enhancement of transformation by SV40 small T antigen (Bikel et al., 1987) is not a complicating factor in our studies.

#### Properties of Cerebellar Cell Lines

Cells from PN2 rat cerebella were placed in culture. After infection and selection, isolated colonies were picked. Cell lines were characterized immunohistochemically with Rat 401, anti-GFAP, and anti-vimentin antibodies (Table 3). All but one of the derived cell lines were Rat

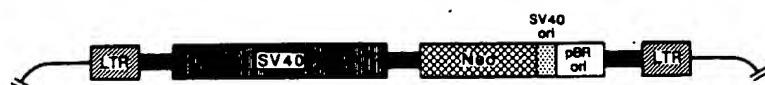


Figure 2. Structure of pZipSVtsA58

The plasmid pZipSVtsA58 was constructed by inserting the genomic BglI-HpaI fragment (nucleotides 5235–2666) derived from the early region of the SV40 mutant tsA58 into the shuttle vector pZipNeo SV(X)1 (Cepko et al., 1984) with BamHI linkers. The fragment was inserted in the sense orientation with respect to retroviral transcription. Although this fragment encodes both the large T and small t antigens, the recombinant retrovirus produced by all the psi2-derived cell lines transduces only the large T antigen (see Results and Figures 3 and 9).

401-positive, and all were vimentin-positive. GFAP expression was found in all of the cell lines derived from *myc* and *neu* infection. In contrast, the SV40 immortalized cell lines were GFAP-negative at the permissive temperature. These results show that it is possible to obtain cell lines expressing markers characteristic of the three proliferating populations defined in Figure 1 and Tables 2 and 3. In addition, they demonstrate that different retroviruses can generate distinct cell lines. The expression of the Rat 401 antigen suggests that the cell lines may be models for CNS precursor cells. Rat 401-positive cells are only transiently present in CNS development, but stable lines expressing this marker can be maintained for months in culture.

### ST15A

To determine whether a Rat 401-positive cell line was capable of further differentiation, one of the SV40 cell lines (ST15A) was grown at an elevated temperature at which the temperature-sensitive T antigen was rapidly degraded (Figure 3). As expected, at the nonpermissive temperature, the cells lose T antigen expression. At the elevated temperature, these cells also lose Rat 401 antigenicity and bind anti-GFAP antibody. At 33°C, Rat 401-negative cells or GFAP-positive cells occur with a frequency of less than  $10^{-3}$ . After several days at 39°C, the majority (95%) of the ST15A cells were Rat 401-negative and GFAP-positive.

An increase in GFAP expression was confirmed by immunoblotting total protein extracted from cells grown at the permissive and nonpermissive temperatures (Figure 4). Immunoblotting also showed that T antigen levels fall at the nonpermissive temperature. Vimentin levels were unchanged in cells grown at the elevated temperature.

However, the large cultures grown to high density necessary for protein chemistry differed from the sparse, analytical cultures used for the immunohistochemical analysis, as Rat 401 expression remained in 25% of the cells after 10 days of growth at the elevated temperature. Differences in cell density or substrate (glass versus plastic) could account for the variation in antigen expression. However, under both conditions, SV40 T antigen was degraded, Rat 401 levels declined, and GFAP levels increased.

ST15A cells also express neuronal features, such as the heavy subunit of neurofilament (Figure 5) and the capacity to generate action potentials (Figure 6) at both the permissive and the nonpermissive temperature for T antigen function. When ST15A cells were grown in N2 medium (Bottenstein and Sato, 1979) for 5 days at 39°C, cells with a neuronal morphology that react with the anti-neurofilament monoclonal antibody were seen (Figure 7). After 9 days in N2 medium, the proportion of SMI 31-positive cells increased (to 90% in some cultures), and cells with very long neurites (greater than 300  $\mu$ m) were present.

As the mechanisms controlling the differentiation of the neuroepithelium may depend on cell interaction, we have studied the differentiated state of ST15A cells when they were cocultured with primary embryonic brain cells. To identify the clonal immortal cells in the presence of primary cells, we first labeled them internally with the succinimidyl ester of fluorescein (Bronner-Fraser, 1985). These internally labeled cells were then analyzed after they were added to primary rat cultures. The primary cells were derived from PN3 cerebellum. The fixed cultures were processed immunohistochemically with anti-T antigen, anti-neurofilament, anti-vimentin, anti-GFAP, Rat 401, and the appropriate secondary antibody. tsA58 immortalized cells were identified by the internal fluorescent label and the binding of antibody established in the same cell. The initial experiments using this paradigm were carried out at 33°C because the red nuclear T antigen fluorescence serves as a control for the internal labeling method. No fluorescein-labeled, T antigen-negative cells were seen. In the presence of primary brain cells, ST15A cells can lose vimentin and Rat 401 antigenicity and gain GFAP reactivity. These data suggest that ST15A cells differentiate into astrocytes in response to signals

Table 3. Antigenic Profile of Cerebellar Cell Lines

Immortalizing Gene	Number of Lines	Primary Antibody		
		Rat 401	Vimentin	GFAP
SV40tsA58	9	+	+	–
	1	–	+	–
v-myc	31	+	+	+
neu	4	+	+	+

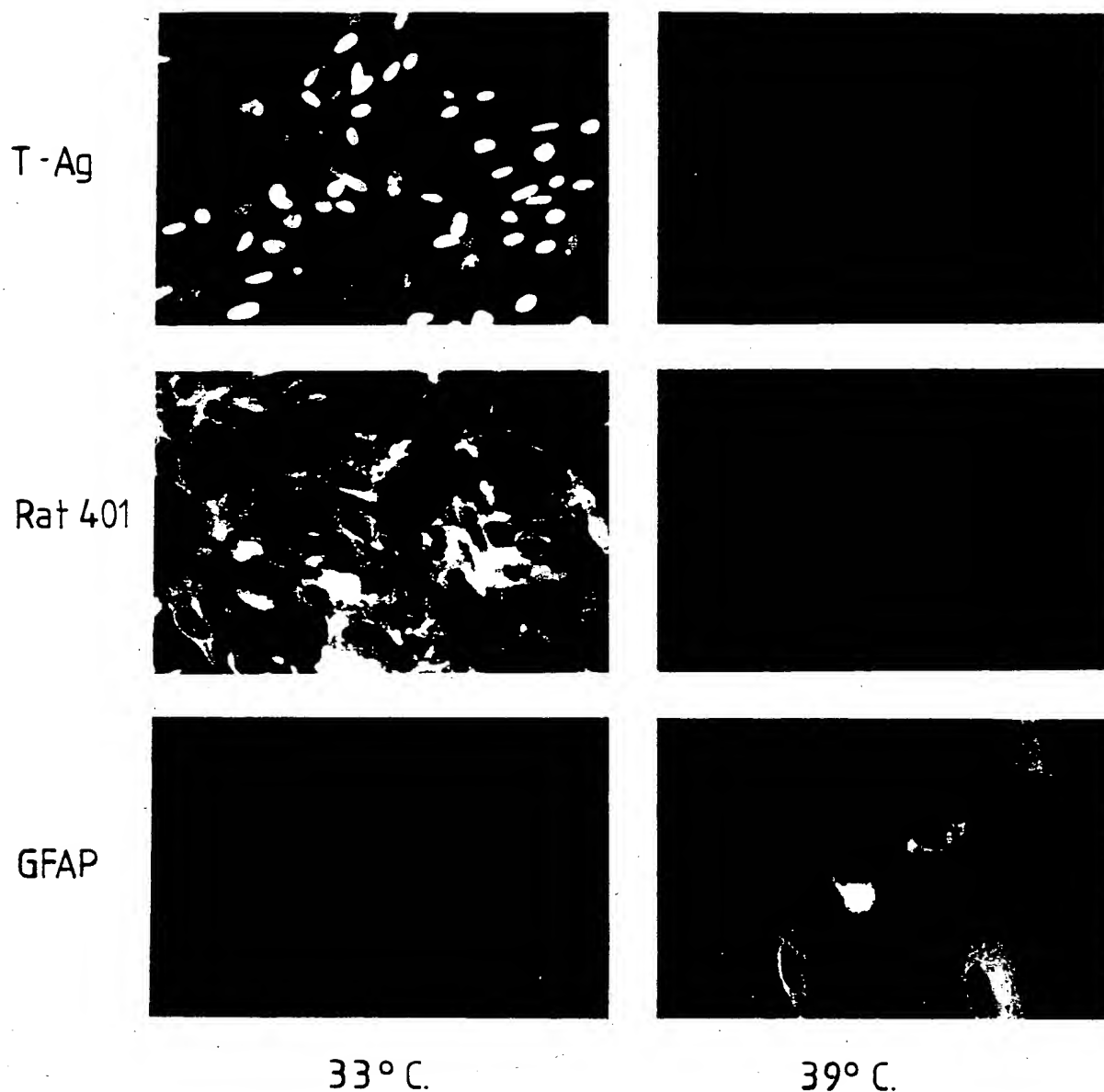


Figure 3. Immunohistochemical Differentiation of ST15A Cells

ST15A cells were grown at 33°C and 39°C. The cells were stained with anti-T antigen, Rat 401, and anti-GFAP antibodies. Immunofluorescence histochemistry shows that T antigen and the Rat 401 antigen are present in all cells at 33°C but are absent from 95% of the cells at 39°C. In contrast, GFAP-positive intermediate filaments are seen at the elevated nonpermissive temperature for SV40 T antigen in 95% of the cells. The time course of this transition is as follows. The anti-T antigen staining disappears after 2 days; the figure shows cells after 3 days at 39°C. Rat 401 staining disappears after 5–8 days in 95% of the cells; the figure shows cells grown at 39°C for 7 days. The expression of GFAP by immunostaining is elevated at 10 days; the figure shows cells stained after 16 days at 39°C.

obtained from primary brain cells. Figure 7 shows ST15A cells in coculture that have extended long processes which stain with anti-neurofilament antibody. These cells with neuronal morphology were clearly different from the flat GFAP-positive cells. The biochemical, morphological, and electrophysiological data show that the ST15A cell line can adopt features characteristic of neuronal and glial differentiation.

#### M15B

The v-myc oncogene generates stable cell lines that express both the Rat 401 antigen and GFAP. The v-myc line M15B was neurofilament-negative when grown in serum and did not generate action potentials. However, M15B cells with neuronal morphology were found when the growth medium contained dibutyl cAMP and retinoic acid (Figure 8). These phase-dense cells charac-



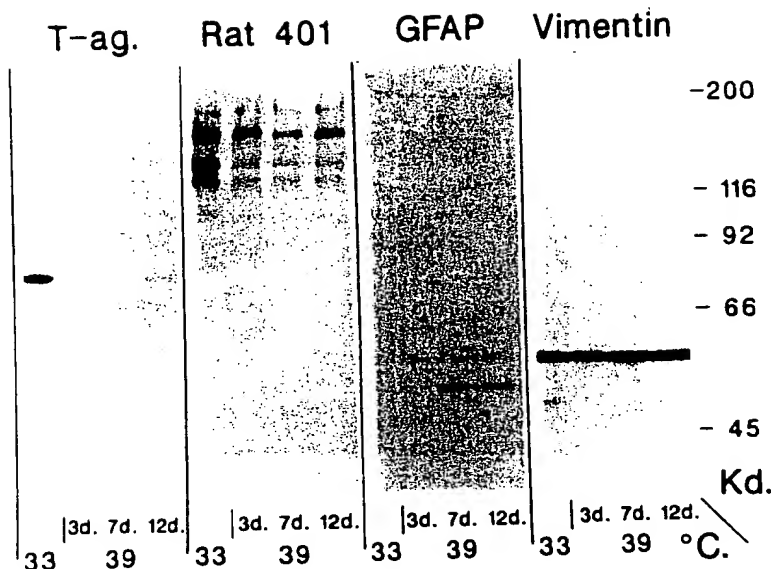


Figure 4. Biochemical Differentiation in the ST15A Cell Line

Western blots of proteins prepared from the ST15A cell line were reacted with anti-T antigen, Rat 401, anti-GFAP, and anti-vimentin antibodies. Four lanes are shown for each antibody. The proteins in the first lane were prepared from cells grown at 33°C, and the three subsequent lanes were prepared from cells grown at 39°C for 3, 7, or 12 days. When shifted to 39°C, T antigen and Rat 401 antigen levels fell, GFAP (50 kd) levels increased, and vimentin (58 kd) levels were unchanged. Total proteins were extracted from the cells by incubation with lysis buffer containing 2% SDS, and the same amount of total protein was loaded in all lanes.

teristically occur in colonies, suggesting that a commitment step which subsequently differentiates neurons occurs in a proliferating cell or that local interactions are important in the morphological change. Two antibodies that stain primary cerebellar neurons (A2B5 and anti-200 kd neurofilament) stain the phase-dense cells but not the flat, GFAP-positive underlying cells. These results show that the v-myc-induced cells can also express neuronal and glial properties.

#### ST15A and M15B Are Clonal Lines

It is clearly important to establish that ST15A and M15B are clonal cell lines. Southern blot analysis was used to determine whether the cell lines were clonal (Figure 9). The size of the SV40 T fragment released by BamHI digestion (designated b in Figure 9A) is too small to encode the small t antigen transcript, and confirms the immunohistochemistry and immunoblotting data (Figure 3; Figure 4) showing that the pZipSVtsA58 retrovirus

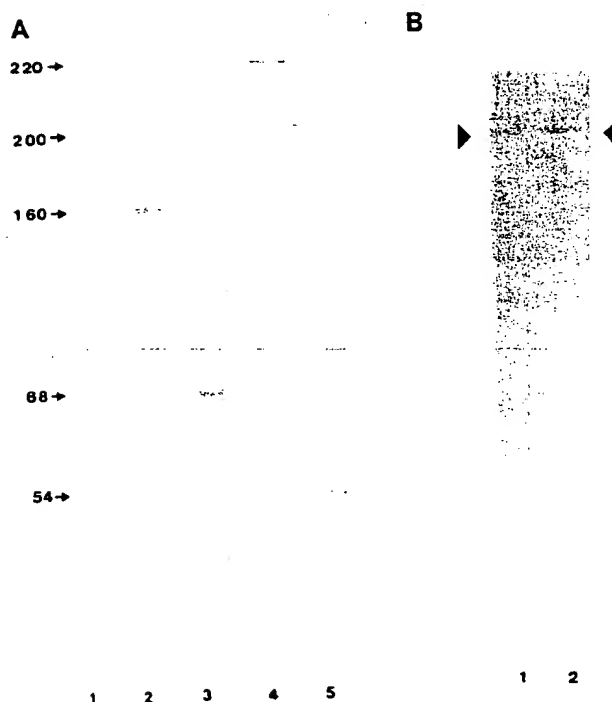


Figure 5. Neurofilament Expression in the Developing Cerebellum and in ST15A Cells  
Western blots of proteins prepared from the PNS cerebellum (A) and ST15A cells (B). Lane A1, anti-200 kd neurofilament; lane A2, anti-160 kd neurofilament; lane A3, anti-68 kd neurofilament; lane A4, Rat 401; lane A5, anti-GFAP; lanes B1 and B2, anti-200 kd neurofilament. The proteins were prepared from ST15A cells grown for 5 days in fetal calf serum containing medium at 33°C (lane B1) and 39°C (lane B2). The 200 kd neurofilament band was present at both temperatures (arrowheads).



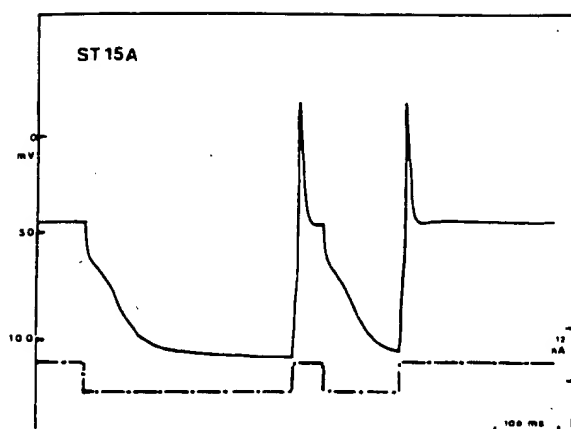
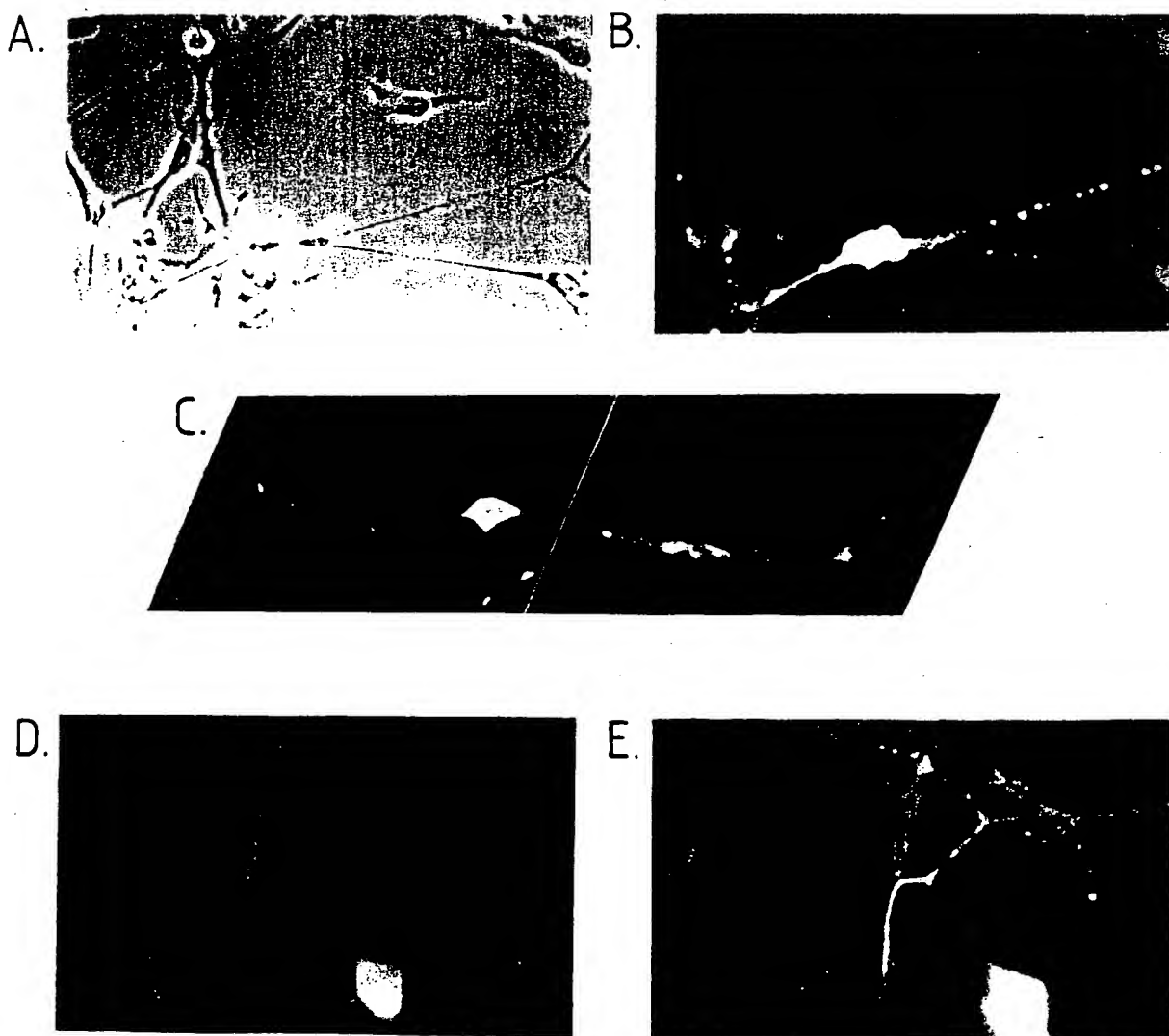


Figure 6. Action Potentials in ST15A Cells  
Intracellular recording was conducted from ST15A cells grown in 10% fetal calf serum, DMEM at 33°C. The action potentials shown occur at anodal break from a hyperpolarizing pulse.

transduces only large T antigen and not small t antigen. XbaI cuts in the viral long terminal repeat sequences, releasing a single full-length proviral sequence from ST15A. EcoRI and BglII cut once in the provirus, yielding only a single fragment in ST15A containing SV40 sequences and flanking cellular DNA. The fact that the EcoRI and BglII bands are both approximately 6.6 kb, the units size of the provirus, might suggest that multiple tandem repeats of the virus exist in ST15A. This interpretation is ruled out by the large BglII fragment obtained in the *neo*-probed Southern shown in Figure 9B. These data show that the ST15A cells carried only a single viral insertion.

In contrast to ST15A, M15B DNA contained two bands complementary to the *neo* probe with all the restriction enzymes tested, suggesting that two independent viral insertion sites are present. We have no explanation for the large size of the XbaI bands. The pattern of fragments seen was stable in multiple subclones of the M15B cell line, showing that it is also a clonal cell line.



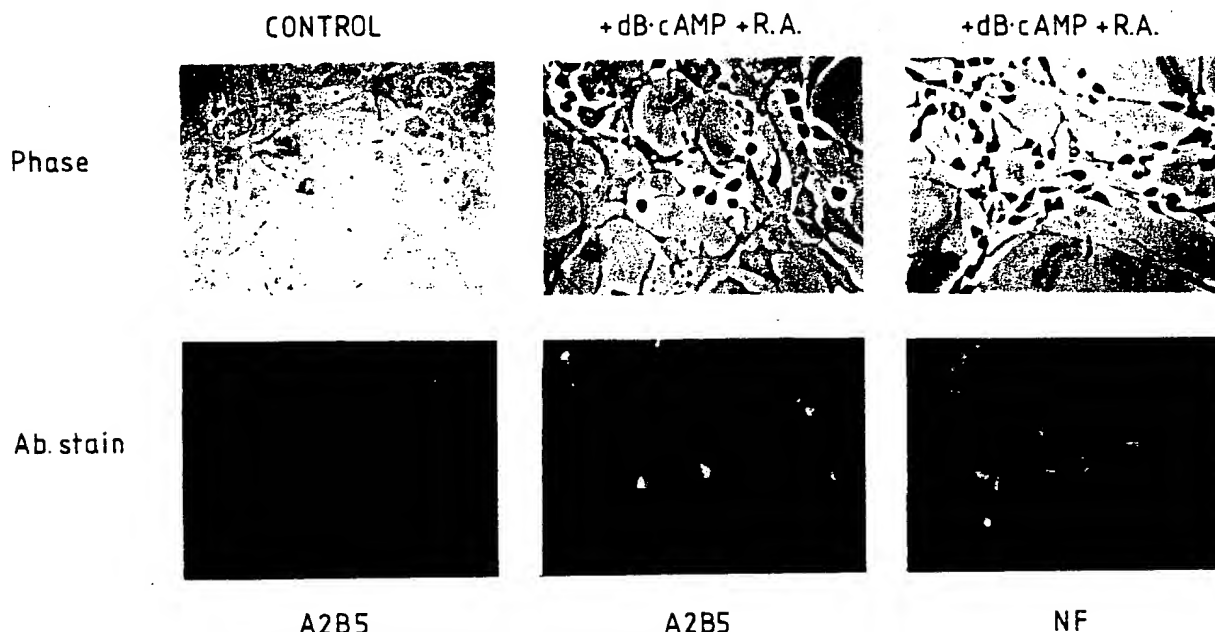


Figure 8. Neuronal Differentiation of the M15B Cell Line

When the M15B cell line is grown in the presence of retinoic acid and dibutyryl cAMP, colonies of cells with small phase-dense cell bodies and long processes appear on the lawn of the flat cells. Morphologically differentiated cells are also biochemically differentiated, expressing the neuronal markers A2B5 and neurofilament ( $\times 25$  objective). M15B cells were grown to confluence in DMEM, 10% fetal calf serum and then treated with  $10^{-7}$  M retinoic acid for 3 days. At this time, the medium was changed to include  $10^{-3}$  M dibutyryl cAMP. After 4 days, cells with neuronal morphology appear in clusters, and after 10 days, up to 20% of the cells have neuronal morphology. The cells with neuronal morphology are A2B5- and neurofilament-positive and GFAP-negative. The A2B5 surface antigen has previously been shown to selectively mark cerebellar neurons in primary culture (Schnitzer and Schachner, 1982). The cells with flat morphology are A2B5- and neurofilament-negative, but remain Rat 401- and GFAP-positive.

## Discussion

The *myc*, *neu*, and T antigen oncogenes can be used to generate Rat 401-positive cell lines from the neonatal cerebellum. The T antigen- and *myc*-derived cell lines grew well in tissue culture over many months and have been serially subcloned. Their differentiation potential has been analyzed. The *neu*-infected cell lines grew very slowly and have not been characterized in detail. Comparison of the antigenic properties of the cell lines with the characteristics of cells *in vivo* and in primary culture suggests that these oncogenes immortalize specific neuroectodermal cell types.

Our results demonstrate that stable cell lines that express markers of transient CNS precursor cells can be made. The two cell lines, ST15A and M15B, that express the Rat 401 antigen also give rise to cells with neuronal

morphology which are neurofilament-positive. The differentiation of these cell lines into neurons is consistent with *in vivo* population measurements which strongly suggest that neurons differentiate from a Rat 401-positive precursor (Frederiksen and McKay, 1988). The ST15A and M15B cell lines can also be GFAP-positive, showing that some genes characteristically expressed in astrocytes can be induced in these cell lines. Our results suggest that these cell lines can express genes that are normally expressed specifically in precursor cells, neurons, or astrocytes.

The ability to obtain neuronal and glial properties in clonal cell lines is consistent with a multipotential stem cell. The presence of multipotential stem cells in some regions of the vertebrate CNS has been demonstrated (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988). Numerous *in vitro* studies also support a

Figure 7. Neuronal Differentiation of the ST15A Cell Line

When ST15A cells are grown in N2 defined medium, many of the cells adopt neuronal morphology (A; phase contrast,  $\times 25$  objective) and react with anti-neurofilament antibody SMI 31 (B and C). ST15A cells also adopt neuronal morphology and antigenicity when they are cocultured with embryonic brain cells in medium containing 10% fetal calf serum for 5 days (D and E). The ST15A cells were prelabeled with the succinimidyl ester of fluorescein; two labeled cells are shown in (D). The binding of SMI 31 antibody revealed with a rhodamine-labeled secondary antibody (E) shows that one of the ST15A cells has extended many fine neurofilament-positive processes, which grow on other cells in the culture, including the undifferentiated ST15A cells. Neuronal differentiation is enhanced at the nonpermissive temperature but also occurs at  $33^{\circ}\text{C}$ .

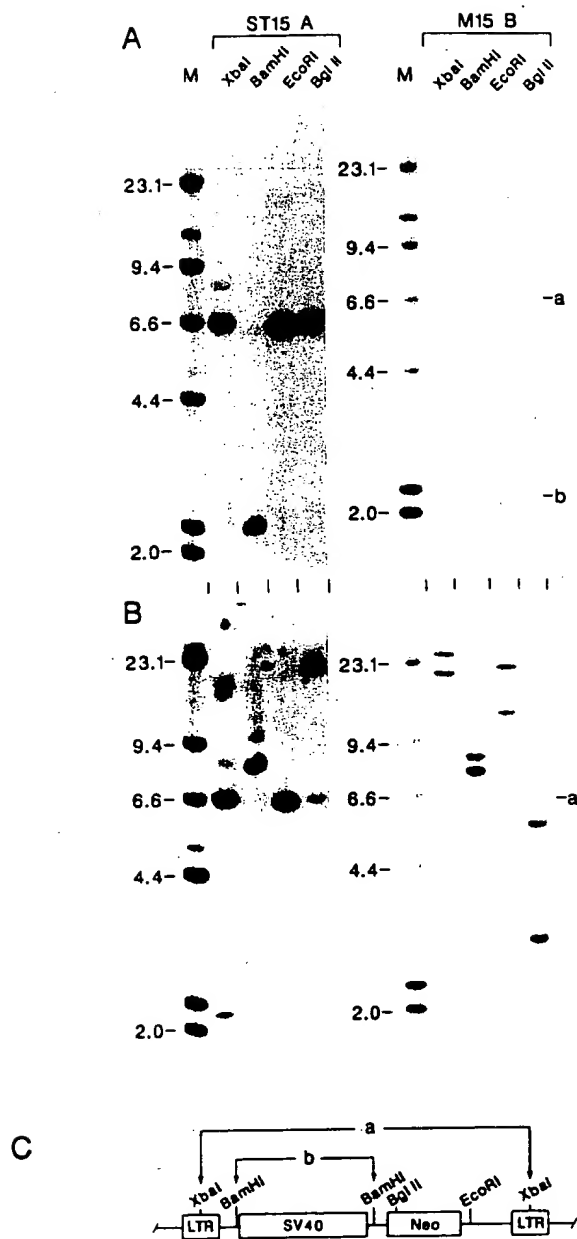


Figure 9. Analysis of Proviral DNA

Ten micrograms of high molecular weight DNA prepared from the indicated cell lines was digested with either XbaI, EcoRI, BglII, or BamHI. The lanes marked M are HindIII-digested bacteriophage lambda DNA. (A) Southern analysis with randomly primed,  $^{32}$ P-labeled fragments specific for the SV40 insert. (B) Analysis of the same filter with neomycin-specific sequences. (C) A schematic of the proviral DNA integrated into the cellular genome. Numbers of the left of the panels show the molecular weights of the lambda HindIII size markers; lowercase letters refer to the size of the viral restriction fragments shown in (C).

common precursor for neuronal and glial lineages (Schubert et al., 1974; De Vitry et al., 1980; Kyritsis et al., 1984; Edde and Darmon, 1985; Schubert et al., 1985). The presence of multipotential precursor cells does not

necessarily imply a single precursor cell type. In fact, our data argue for at least three different proliferating populations in vivo. The antigenic profiles of the immortal cell lines ST15A and M15B suggest that these cell lines may be homologous to different in vivo proliferating populations. Additional experiments are needed to explore this possibility, but the in vitro data presented here suggest that the cell lines immortalized with a conditional oncogene will allow further analysis of cell lineage mechanisms in the developing cerebellum. The use of temperature-sensitive oncogenes may also be useful in vivo. As the core body temperature of rodents is nonpermissive, we can envisage experiments in which immortal cells will differentiate after transplantation into a developing host. Experiments of this kind may bridge the gap that currently exists between data from in vivo lineage experiments and in vitro studies with cell lines.

#### Experimental Procedures

##### Animals, Cell Lines, and Antibodies

Sprague Dawley rats were obtained from Taconic Inc., NY. NIH 3T3 cells and psi2 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) calf serum, penicillin, and streptomycin. The anti-vimentin antibody was from ICN (Cat. No. 69-127), the anti-GFAP antibody was from ICN (Cat. No. 69-110), the anti-neurofilament antibody was from Sternberger-Meyer (Cat. No. SMI 31). Additional anti-neurofilament antibodies were obtained from Sigma. The Rat 401 antibody was established in this lab and has been previously described (Hockfield and McKay, 1985), the anti-T antigen antibody was monoclonal antibody 412, prepared by E. Harlow. In double label experiments, the rabbit anti-vimentin was from R. Hynes, the rabbit anti-GFAP was from L. Eng, and the rabbit anti-neurofilament was purchased from ICN (Cat. No. 20074). Secondary antibodies were obtained from Cappel-Worthington.

##### Analysis of Cerebellar Cell Types In Vivo

A detailed description of dissociation protocols, autoradiographic methods, and control experiments for cell numbers and staining procedures can be found in Frederiksen and McKay (1988). The cerebellum was dissected from postnatal rats, and the cells were dissociated by trituration after digestion in 0.15% trypsin in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate buffered saline. Measured aliquots of the dissociated cell suspension were spun onto coverslips and stained with primary antibodies and rhodamine- or peroxidase-conjugated secondary antibodies. The data shown were derived from four or more animals from two or more litters.

##### Primary Cultures and Infection Protocols

The cerebellum was removed from PN2 animals and incubated in 0.08% trypsin for 30 min at 37°C. After further dissection into small pieces, the cells were dissociated by trituration in DMEM, 10% fetal calf serum using a Gilson 1 ml Pipetman. The cell suspension was plated onto polyornithine-coated tissue culture dishes (15  $\mu\text{g}/\text{ml}$ , Sigma) in DMEM, 10% fetal calf serum and incubated at 37°C. Twenty-four hours after plating, the cells were infected for 2 hr with the recombinant retroviruses in 8  $\mu\text{g}/\text{ml}$  polybrene (Aldrich). After infection, the virus-containing medium was replaced with fresh DMEM, 10% fetal calf serum. tsA58-infected cell lines were subsequently grown at 33°C. Forty-eight hours after infection, the cultures were passaged and subjected to selection in 200  $\mu\text{g}/\text{ml}$  G418 (Geneticin, Gibco). The selective medium was changed every 3–4 days. Within 3 weeks, control uninfected dishes had very few remaining cells and the infected dishes had macroscopic G418-resistant colonies. Colonies were picked using cloning rings and expanded into 96-well plates.

# Growth and Characterization of Cell Lines

The cells were continually grown in DMEM, 10% fetal calf serum. The *neu*-infected cells could be expanded and frozen but always grew slowly. The *v-myc*- and T antigen-derived cells grew rapidly for a year and were subcloned. The immunohistochemical analysis of antigen expression in ST15A cells was carried out in DMEM and selected batches of fetal calf serum at 33°C and 39°C. The immunoblotting procedure was as described by Towbin et al. (1979). Analysis of proviral DNA was carried out on high molecular weight DNA prepared and fractionated on 0.8% agarose gels. The DNA was transferred to Zeta Bind (CUNO Labs, Meriden, CT) and hybridized by standard methods (Southern, 1975; Maniatis et al., 1982). The serum-free medium used was the N2 medium of Bottenstein and Sato (1979). The cocultures were carried out after labeling the immortal cells with fluorescein succinimidyl ester (Bronner-Fraser, 1985). These labeled cells were then added to primary cultures of E14 cerebral cortex or PN2 cerebellum 1 day after the primary cells were plated. Electrophysiological data were obtained with conventional intracellular recording methods using a 70 MΩ, 4 M potassium acetate electrode.

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